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GRANT NUMBER DAMD17-96-1-6003

TITLE: Improvement of Cultured Keratinocyte Grafts for Burn Wounds

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REPORT DATE: January 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1997	Annual (31 Dec 95 - 30 Dec 96)

4. TITLE AND SUBTITLE
Improvement of Cultured Keratinocyte Grafts for Burn Wounds

5. FUNDING NUMBERS
DAMD17-96-1-6003

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Chapel Hill
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

During the first year of grant support all components in the Statement of Work scheduled for year one have been completed, with one still in progress. The results have demonstrated that cultured keratinocyte allografts are less immunogenic in burned animals as measured by priming for second-set rejection and cytotoxic lymphocytes. Burn injury impairs expression of alloantigen on keratinocyte allograft as one mechanism of this effect. A second mechanism that effects the response is an increase in cytokine in the burn wound and distant normal tissue after burn injury. Notably, cultured keratinocyte allografts deficient in Class II histocompatability antigen are significantly less immunogenic, especially after burn injury, and may provide a means to produce universal donor keratinocytes.

14. SUBJECT TERMS
Burns, keratinocyte grafts

15. NUMBER OF PAGES
65

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Anthony A Meyer 1/30/92

PI - Signature Date

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INTRODUCTION:

This is the first annual report on Grant DAMD17-96-1-6003, entitled **"Improvement of Cultured Keratinocyte Grafts For Burn Wounds"**.

This grant is designed to study the potential use of cultured keratinocyte allografts for burn wound coverage. Burn wounds represent a significant problem to military and civilian personnel. In significant military injuries, burns are present in approximately 10% of casualties. The limiting factors in recovery from burn wounds are the size and severity of the burn injury and the time required to cover the burn wound. Presently, with burns greater than 50% total body surface area (TBSA), there is a significant delay in burn wound coverage with accompanying increased risk for infection causing death or serious morbidity.

The factors which presently limit early burn wound coverage are the size of the burn and availability of donor sites. Cultured keratinocytes from an individual patient can be grown in a laboratory, but there is a delay of three to five weeks for even a small amount of grafts and the potential for loss of grafts due to laboratory accident makes this a less than reliable source of skin replacements. Cultured keratinocyte allografts, that is skin from another individual grown and kept in culture, have been used but with limited success because of late rejection.

The purpose of this study is to investigate the mechanisms involved in recognition and rejection of cultured keratinocyte allografts and to potentially develop means by which this response can be suppressed either by modification of the immune response or by modification of the keratinocytes which would render them less immunogenic.

The scope of this research is to perform experiments in rodent models to determine the mechanisms involved in rejection and to see if genetically altered keratinocytes can be grown successfully in culture and transplanted to animals. The immunogenicity of these altered keratinocyte grafts will be studied with the purpose of trying to produce means of altering the immunogenicity of keratinocytes without significantly effecting their biologic function.

This research is a follow-up to previous grant, DAMD17-91-Z-1007. In these studies and other studies done in our laboratory, we have demonstrated the following factors which have led us to our present research.

(1) Cultured allogeneic keratinocytes can be successfully grafted in mice with at least short term graft survival.(1)

(2) Cultured keratinocyte allografts express MHC Class II histocompatibility antigen in less than 24 hours after grafting, but this effect decreases with time.(2)

(3) The amount of MHC Class II antigen expression is decreased in animals with burn injury.(3)

(4) Although CK allografts do not cause the host to produce cytotoxic antibody as assessed by mixed lymphocyte response, CK allografts do sensitize recipients as measured by accelerated second-set rejection and increased cytotoxic T-lymphocytes.(1,2)

(5) The sensitization by CK allografts is significantly decreased in burned animals and the magnitude of the decrease appears related to burn size.

(6) NIH Swiss Albino 3T3 fibroblasts, growth arrested with mitomycin C and used in co-culture with keratinocytes, persist in final keratinocyte grafts. (4,5)

BODY:

The following review of the first year of the grant follows the format in the statement of work on page 30 of the initial proposal. The time line on page 31 indicates that the first year of the grant should include or address sections A1-1, A1-2, A2-1, A2-2, A2-3 and B. These will be identified separately and either the completed manuscript that describes the research will be included, or the specifics of the methods, results, and discussion will be provided.

A.1. ***Determination of effect of burn size on sensitization and allograft antigen expression. STEP 1: Assess response to second set rejection and development of cytotoxic lymphocytes in animals with different sizes of burn wounds. This will take approximately three months to perform.***

Briefly, in these studies animals received burn injury of 20% and 40% of total body surface area (TBSA). These animals were compared with non-burn controls. The results and methods are summarized in the first paper in the Appendix entitled "1995 Moyer Award. The Effect of Burn Injury on Allograft Rejection, Alloantigen Processing, and Cytotoxic T-

Lymphocyte Sensitization".(6) This paper describes the methods summarized in the initial proposal. The results shown in Table I from this manuscript demonstrate that burn injury suppresses the rejection of initial allograft and has an even more dramatic effect on the second-set rejection of allograft. This is demonstrated graphically in Figure 1 in the same paper.

The effect of the various size burn injury on cytotoxic lymphocyte function is shown in Figures 2-5, which demonstrates the evolution of cytotoxic lymphocyte suppression in days 3-6 in culture. This data demonstrates clearly that the immunosuppressant effects of burn injury are burn size related. The implications of this are that people with excessively large burns may have very limited ability to recognize allograft and respond with either primary or second set rejection. This supports previous clinical observation of prolonged graft survival of human allograft, and demonstrates that the second set rejection is even more suppressed.

A.1. STEP 2: ***Graft biopsies to measure donor graft antigen expression taken during Step A.1 will be extracted and frozen and western blot performed on all samples at the conclusion of Step 1. This will take approximately one month.***

The studies outlined in Step 2 were done and their results are summarized in paper 2 in the Appendix. This paper, entitled "The Relationship Between Interferon Gamma and Keratinocyte Antigen Expression After Burn Injury"(2), describes the effects of burn injury on expression of Class II histocompatibility antigen. The methods of burn injury, wound biopsy and Western immunoblotting are outlined in the manuscript. The results shown in Figures 6 and 7, clearly demonstrate that burn injury suppresses Class II histocompatibility antigen production in a manner directly related to the area and depth of the burn injury. Burns of 40% reduce Class II histocompatibility antigen production to less than 20% of normal control. Although animals with the 40% full thickness burn had elevated interferon gamma in their serum, the levels were dramatically decreased in the burn wound itself where allograft production of antigen would occur. It has been suggested that interferon gamma levels may be associated with Class II histocompatibility antigen expression in the area of the burn. This relationship between interferon gamma and Class II histocompatibility antigen has been demonstrated by Aubock (8) in previous studies. These results suggest that one of the mechanisms by which the delayed second set rejection of allografts and CTL production seen in the burn animals noted in Step 1 may represent decreased immunogenecity of the keratinocyte allografts because of decreased

amount of antigen expression. Potentially, future studies could examine the specific mechanisms that control antigen production as a way to induce tolerance to keratinocyte allografts in patients with large burns.

A.2. ***Determination of mechanisms responsible for impaired second set rejection and CTL response after burn injury. STEP 1: CTL response will be measured selectively after depleting lymphocyte cell populations. This will take approximately four months.***

Mouse spleen cell populations from animals with 20% full thickness flank burns, as documented in the methods of papers 1 and 2 in the Appendix (6,7), were assessed for different lymphocyte cell populations. Briefly, spleen cells were isolated as they are for the CTL assays, red cells were removed, and the resultant lymphocyte preparations were incubated for a period of 20 minutes on ice with fluorescent labeled antibodies to different cell population markers. These antibodies and their specific label and target antigen are listed in the table below. All antibodies were obtained from PharMingen, San Diego, CA.

Tag	Receptor	Cell
FITC (Fluorescein Isothiocyanate)	CD8	CTL
Cy-Chrome™ (Red 670)	CD4	T-Helper
PE (R-phycoerythrin)	CD25	Memory (IL-2 Receptor)

These cells are then aspirated into the FACScan flow cytometer (Becton Dickinson) at the University of North Carolina Flow Cytometry Facility and the number of cells in each sample counted. The relative numbers are corrected for the total number of cells and percentages calculated. Splenocytes from burn animals are compared to normal sham animals and expressed in the Figures 8 and 9. Statistical analysis is done by unpaired T-tests and the p-value included in the tables attached to Figures 8 and 9. In Figure 8, three days post burn injury there is a significant decrease in CD-4 cells in the burned animals. By 14 days after burn injury, however, there is an increase in CD-8 cells and a further reduction in CD-4 cells. This increase of CD-8 cells may indicate why cytotoxic lymphocyte function may recover by two weeks after burn

injury, leading to subsequent graft rejection. The implication of this is that survival of cultured keratinocyte allografts after burn injury may be prolonged by continued suppression of the CD-8 population.

A.2. STEP 2: ***Second-set rejection and CTL response in mice receiving grafts deficient in MHC Class I and Class II antigens will be measured. This will take approximately four months but will run concurrently with A.2. Step 1.***

Preliminary studies in mice deficient in Class I or Class II MHC histocompatibility antigens were done and are in the third article in the Appendix, "Allogeneic Keratinocytes Deficient in Class II Antigens Failed to Prime the Host For Accelerated Second-Set Rejection or Enhanced T-Lymphocyte Cytotoxicity" (9). Briefly, Class I and Class II major histocompatibility deficient C57BL/6 (B6) mice were studied for their ability to prime for second-set rejection of B6 allograft and were tested for their immunogenicity by cytotoxic lymphocytes as described in the identical methods used in A.1. Step 1. The data shown in Table II and Figure 10 in the Appendix clearly demonstrate that Class I deficient and Class II deficient animals have less ability to prime animals for accelerated second-set rejection. Furthermore, they are less effective at generating late CTL response to keratinocytes than are the full thickness grafts in the knock-out animals themselves. This data demonstrates that cultured keratinocytes from animals deficient in Class II histocompatibility antigens do have limited immunogenicity to induce second-set rejection or cytotoxic lymphocytes. The implication of this data is very important and it may identify that human keratinocyte cell lines could be developed that are deficient in the histocompatibility antigens necessary to trigger rejection. It may be possible to develop a clone of human keratinocytes that are not rejected and would serve as a "universal donor" keratinocyte population for burn wound coverage and potentially other clinical uses.

A.2. STEP 3: ***Individual cytokine enhanced supplementation and/or depletion will be accomplished by selectively adding or blocking cytokines using monoclonal antibody to specific cytokines. This will take approximately four months.***

Initial studies have been generated to examine the cytokine changes in burn wound, serum and normal tissue after burn injury. Identifying the patterns of suppressive and stimulatory cytokines in these models will help us determine which cytokines may be appropriately manipulated in in vitro and in vivo models to suppress cytotoxic lymphocytes. These studies

were done according to the methods described in the initial proposal. Briefly, CBA mice were given a 20% full thickness flank burn using the methods described in the previous papers. At time points of 1 hour, 6 hours, 24 hours, 72 hours, and 168 hours after injury, blood was sampled from the retro-orbital plexus and tissue below the level of the eschar and from normal skin were taken and processed. Briefly, the serum was separated from the blood samples and frozen. The tissues were sonicated in HBSS and centrifuged at 2500 rpm for five minutes and the supernatant saved. The serum and supernatant were then tested using standard ELISA kits obtained from BioSource International, Camirillo, CA, and PharMingen, San Diego, CA. The data for IL-2 is shown in Figure 11, IL-4 is shown in Figure 12 and interferon gamma is shown in Figure 13. Additional cytokines are in the process of being studied.

These data demonstrate that all three cytokines are elevated in the normal tissue of burned animals compared to sham animals. Furthermore, all three cytokines are elevated in tissue below the eschar compared to normal tissue from burned animals. This effect lasts at least one week.

B. ***Determination if allogeneic and xenogeneic passenger antigen cause rejection. Step 1. Animals will be immunized by tail graft or IP injection and tested for rejection of cultured keratinocyte autografts grown in the presence of these cultured components. Cultured keratinocyte allograft rejection will be assessed. This will take approximately four months and will run concurrent with steps A1. and A.2.***

This study was done to determine whether or not the passenger fibroblasts in cultured keratinocytes prime animals for graft rejection. This data is summarized in the paper entitled "Allogeneic Fibroblasts Used To Grow Cultured Epidermal Autografts Persist in Vivo and Sensitize The Graft Recipient for Accelerated Second Set Rejection" (5) and is paper 4 in the Appendix. Briefly, this study examined cultured keratinocyte autografts grown in the presence of allogeneic fibroblasts which were used to prime recipients. Tail grafts of the haplotype of the allogeneic passenger fibroblast were then assessed for second-set rejection. This data shown in Figure 14 of this paper clearly demonstrates that animals primed by the fibroblasts given intraperitoneally are sensitized for accelerated second set rejection. The subsequent studies demonstrated in Table III and in Figure15 again clearly demonstrate that autologous keratinocytes grown in the presence of allogeneic fibroblasts prime those animals for accelerated second-set rejection of tail grafts of the haplotype of the allogeneic fibroblast. This data clearly

points out that cultured keratinocyte grafts prepared in many centers that grow cultured skin, (including those that are commercially available) include passenger fibroblasts, despite efforts being made to reduce them. Furthermore, the fibroblasts that persist, although very few in number, are sufficient to prime the animals for late graft rejection. The implication of this data is that the use of passenger fibroblasts that are allogeneic or xenogeneic in comparison to the donor can lead to the late graft loss described in this paper and clinically.

CONCLUSIONS:

These results in this first year report follows the statement of work and clearly show significant progress in the pursuit of the goals of the grant. Specifically, the following conclusions can be drawn based on this data:

(1) Burn injury suppresses host ability to mount an allograft reaction, and this suppression is directly proportional to burn size. The implications of this are that those individuals in most need of keratinocyte allografts may in fact be the ones in whom keratinocyte allografts may persist the longest or may in fact be permanently accepted if tolerance can be induced.

(2) The production of Class II histocompatibility antigen by keratinocyte allografts is significantly reduced when these are placed on burn recipients. The implications of this finding is very important in that it may identify a mechanism by which the immunogenicity of allografts can be reduced to promote graft survival and possibly induce a state of tolerance.

(3) Burn injury has significant effects on lymphocyte sub-populations and these effects change over a period of several weeks following burn injury. The implication of this is that the identification and manipulation of sub-populations responsible for allograft recognition and destruction may in fact be another mechanism by which prolongation of graft survival may be achieved.

(4) Cultured keratinocyte allografts deficient in Class II histocompatibility antigens are less immunogeneic and may survive for a greater period of time than their normal counterparts. This is especially important because it identifies a possible strategy by

which keratinocytes may be rendered deficient of various components of their histocompatibility antigens. This, in turn, could produce a "universal donor" cultured keratinocyte with which patients could be grafted to produce a long term and possibly permanent graft survival.

(5) Initial studies of cytokine response following burn injury show differences that suggest that manipulation of some of these cytokines may permit altering the immunogenicity of the allograft or the responsiveness of the host to the alloantigens. Further studies of other cytokines and interactions to these cytokines will be done if the grant is continued in an attempt to identify which cytokines effect the greatest prolongation of allograft survival.

(6) Allogeneic fibroblasts used in growth of cultured keratinocyte grafts have been demonstrated to prime recipients to subsequent graft rejection and can accelerate graft rejection if they are of a different haplotype than the keratinocytes themselves. The implication of this is that methods to rapidly grow differentiated keratinocyte allograft sheets in the absence of feeder layer fibroblasts need to be developed, or that means to eliminate the immunogenicity of these feeder layer fibroblasts need to be developed.

In summary, this data demonstrates that cultured keratinocyte allografts may be of significant clinical use in the future. Work that needs to be done to identify this are to look at different genetically altered cell lines that are deficient in histocompatibility antigens and to attempt to manipulate the environment of keratinocyte allograft recognition and host response in order to limit or eliminate graft destruction. In order for these next developments to be considered, the grant would have to be refunded or considered for re-submission.

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APPENDIX 1: TABLES

Table I. Effect of burn injury and priming on the survival of second-set tail allografts.

	Primed			**Unprimed**		
	Primary flank graft: allograft			Primary flank graft: autograft		
	0% Burn	20% Burn	40% Burn	0% Burn	20% Burn	40% Burn
MST, days	9	10	12.5	13	14	15
MST, range	7-11	8-12	10-16	10-15	12-17	13-18
Number	18	16	16	15	17	13

Mice receiving primary flank allografts are defined as primed, whereas mice receiving primary flank autografts are defined as unprimed. MST, Median survival time of secondary tail allografts.

Table II. Median survival time of second-set tail allografts (days).

Flank graft	B6 allograft	I-/- B6 allograft	II-/- B6 allograft	CBA autograft
CK	9	11*	12*	13*
FT	9	9	11*	

*p<0.05 vs B6 FT.

Table III. Median survival times of second-set tail allografts.

	Control Groups			Cultured Epidermal Autografts		
	NIH FT Allograft ($H\text{-}2^q$)	CBA FT Autograft ($H\text{-}2^k$)	3T3 IP ($H\text{-}2^q$)	CBA CEA +3T3 ($H\text{-}2^q$)	CBA CEA -3T3 ($H\text{-}2^q$)	CBA CEA +LTK ($H\text{-}2^k$)
MST, days	9*	12	9.5*	9*	10*	12
MST, range	7-11	10-14	7-12	7-11	7-12	10-14
N	16	15	16	10	15	13

MST, median survival time; N, number per group; FT, full-thickness; IP, intraperitoneal; CEA, cultured epidermal autografts. *$p<0.01$ vs CBA FT, CEA+LTK groups.

APPENDIX 2: FIGURES

Figure 1

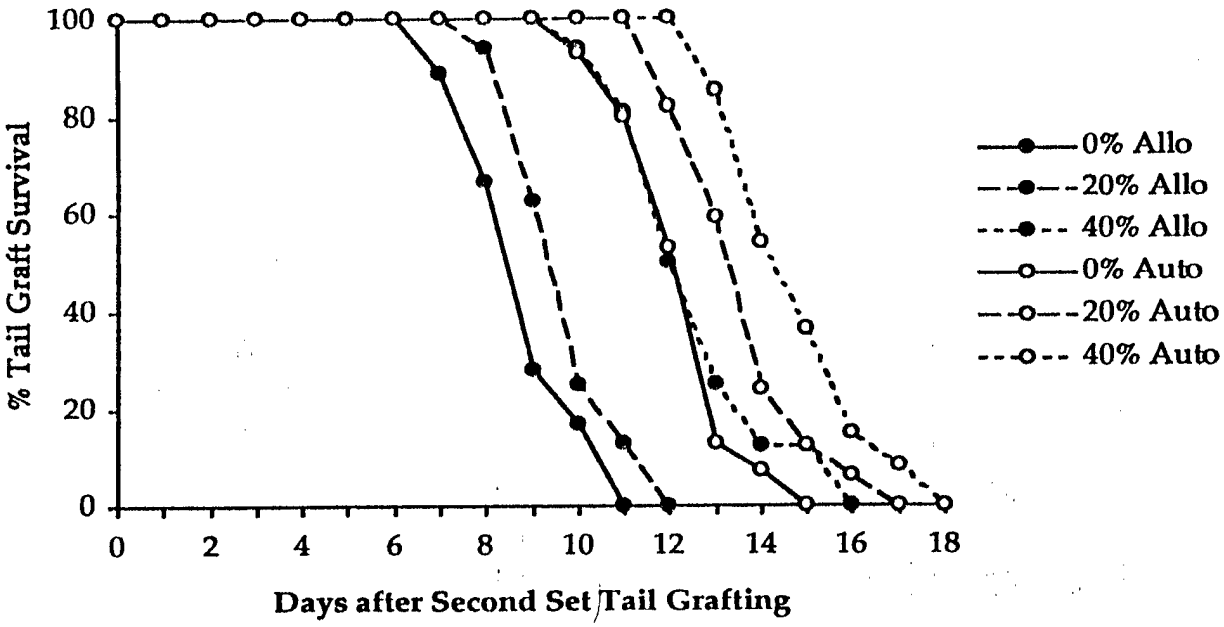


Figure 1. Survival curves of secondary tail allografts depicting effect of thermal injury and priming on second-set rejection. Burn injury significantly prolonged second-set tail graft survival in both the primed *(Allo)* and unprimed *(Auto)* groups ($p < 0.05$). Sensitized rejection was impaired to a greater extent than naive rejection.

Figure 2

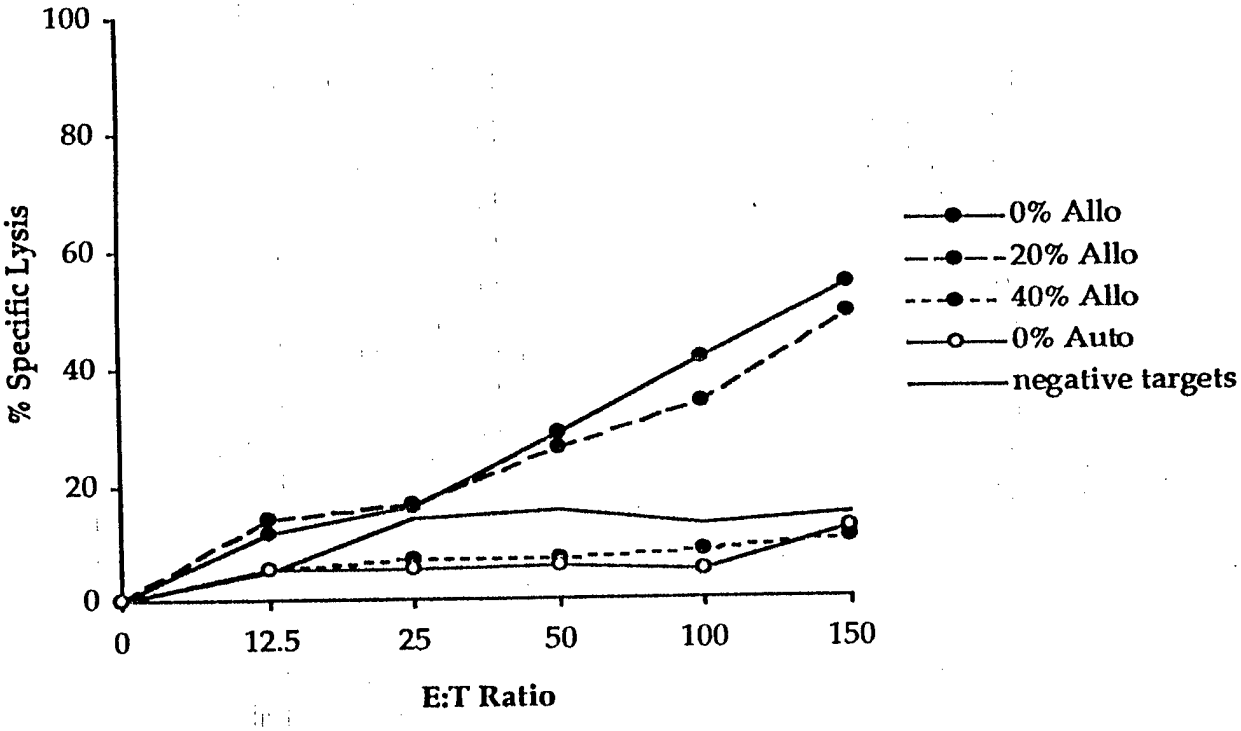


Figure 2. Effect of burn injury on CTL alloreactivity 3 days after in vitro stimulation.

Figure 3

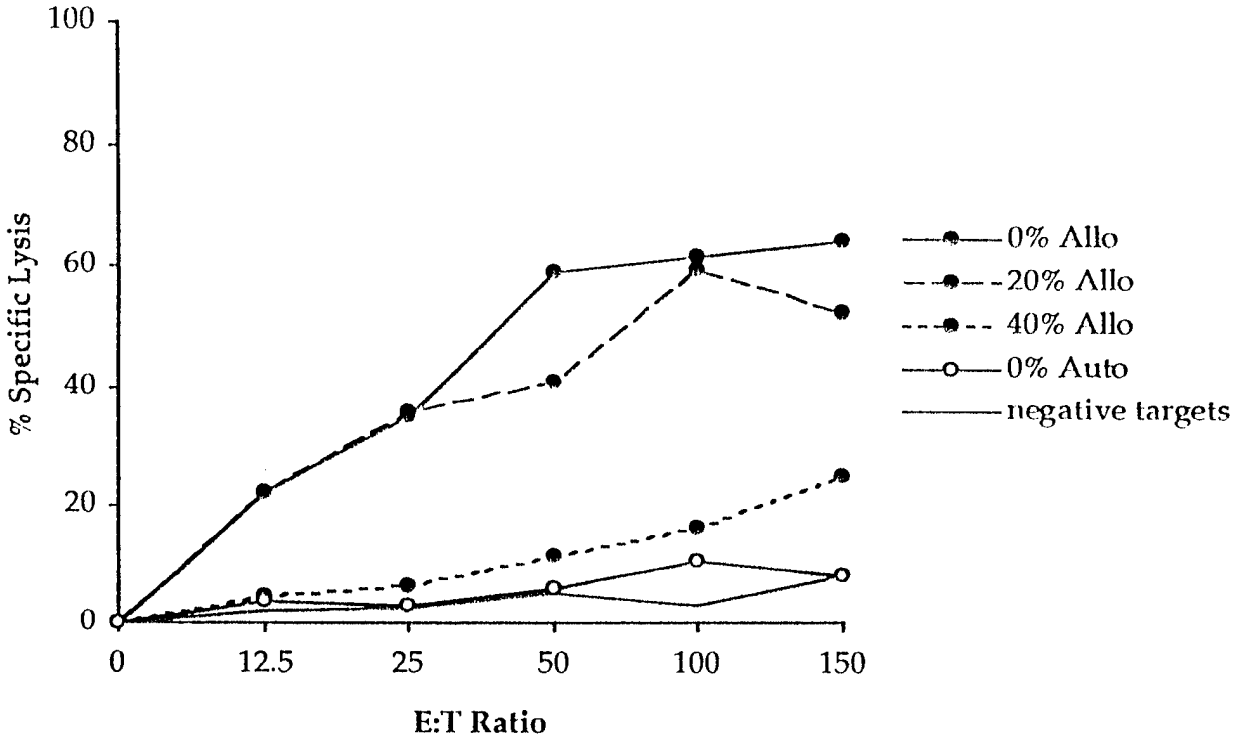


Figure 3. Effect of burn injury on CTL alloreactivity 4 days after in vitro stimulation.

Figure 4

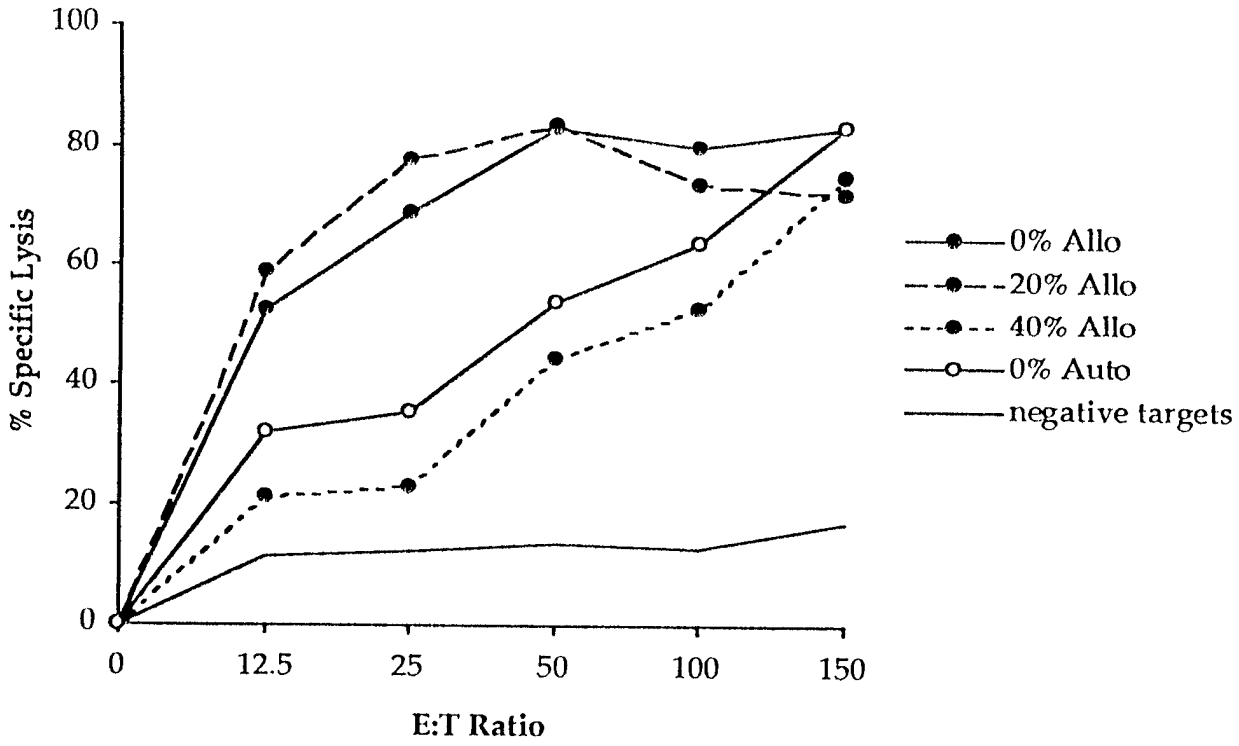


Figure 4. Effect of burn injury on CTL alloreactivity 5 days after in vitro stimulation.

Figure 5

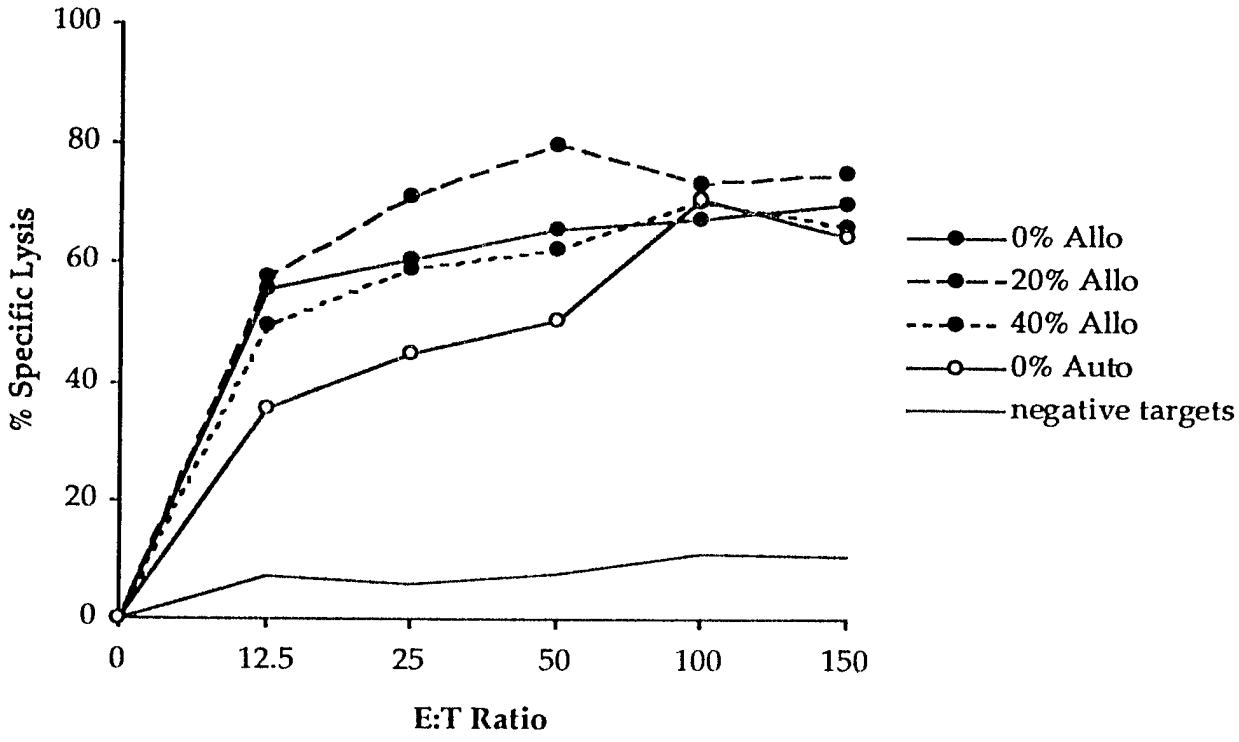


Figure 5. Effect of burn injury on CTL alloreactivity 6 days after in vitro stimulation.

Figure 6

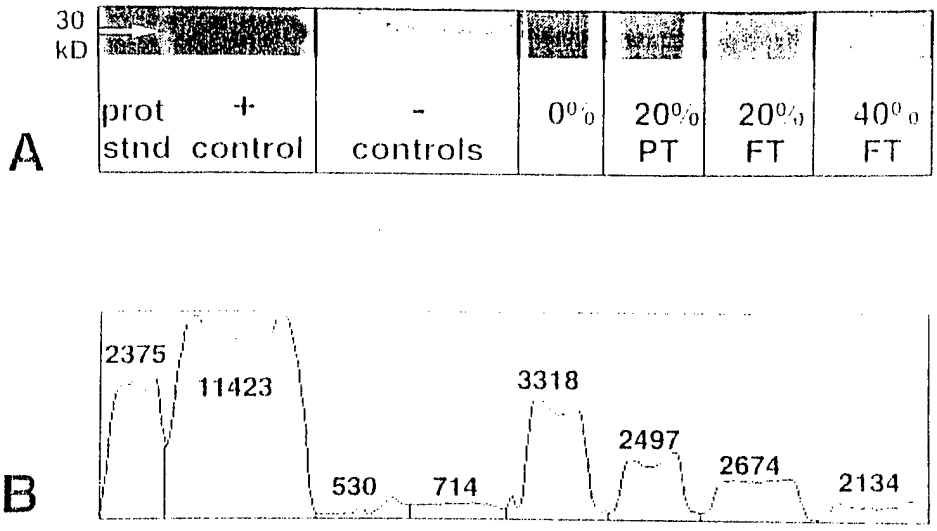


Figure 6 (A) Western immunoblot depicting MHC class II alloantigen expression after burn injury and grafting. prot stnd: low molecular weight protein standards; + control: C57BL/6 splenocytes; −controls: CBA splenocytes and CBA skin; 0%: sham burn; 20% PT: 20% total body surface area partial-thickness burn; 20% FT: 20% total body surface area full-thickness burn; 40% FT: 40% total body surface area full-thickness burn. (B) Video densitometrogram relating absolute pixel density of individual protein bands. Burn injury decreased alloantigen expression as burn size increased.

Figure 7

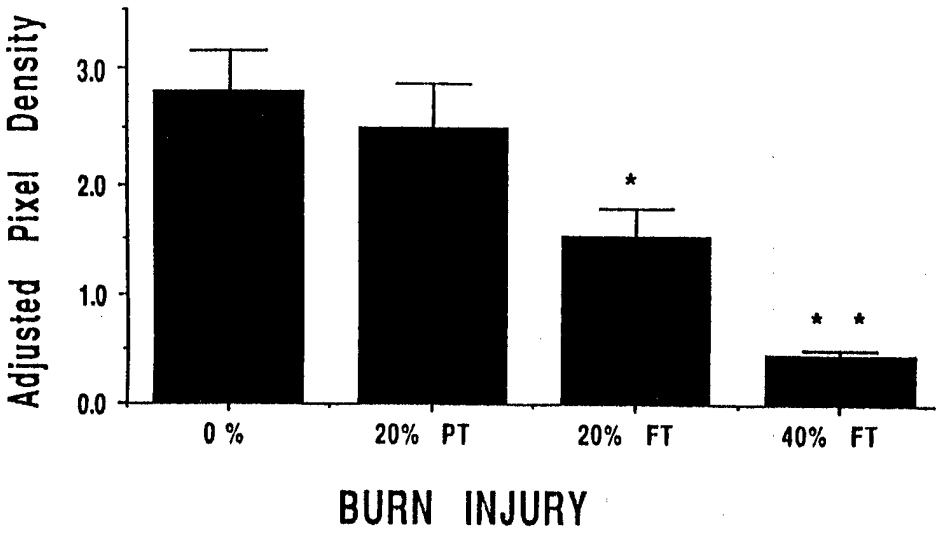
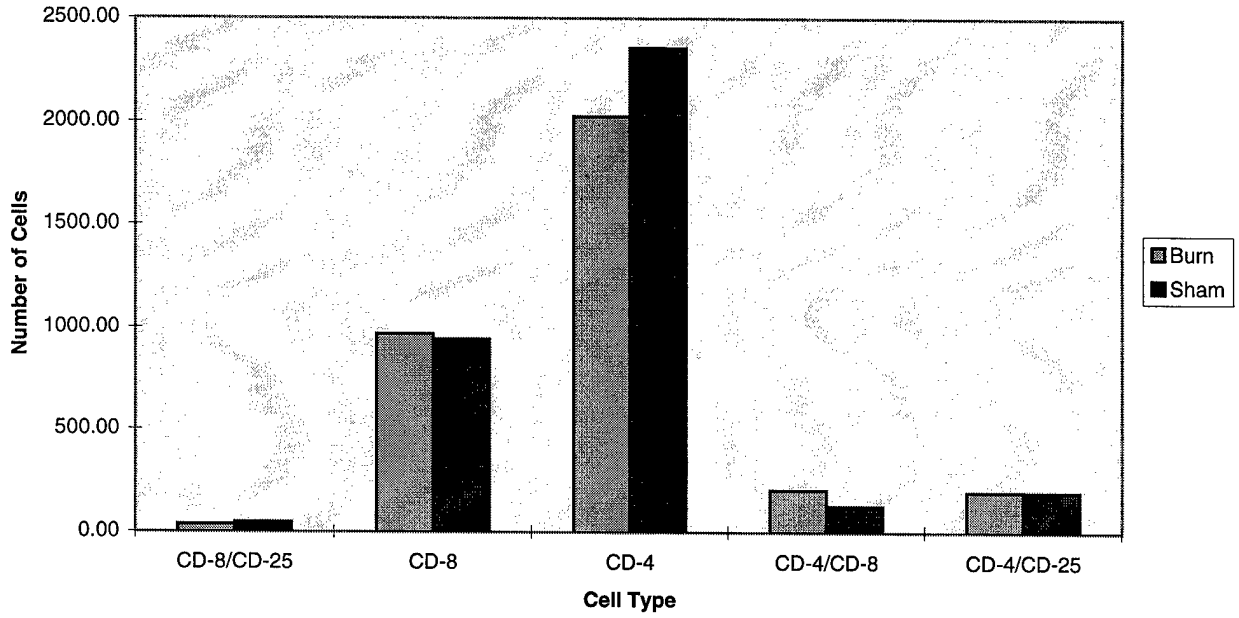


Figure 7 The effect of burn size on MHC class II antigen expression in CK allografts 3 days after excision and grafting. Each group contains approximately seven surviving animals. Immunoblots were scanned with video densitometry to quantify alloantigen expression, which is depicted graphically as mean adjusted pixel density. Error bars represent standard error of the mean. Both 20% FT and 40% FT burn injuries significantly inhibited class II alloantigen expression. *$p < 0.05$ *vs.* 0%; **$p < 0.001$ *vs.* 0%.

Figure 8

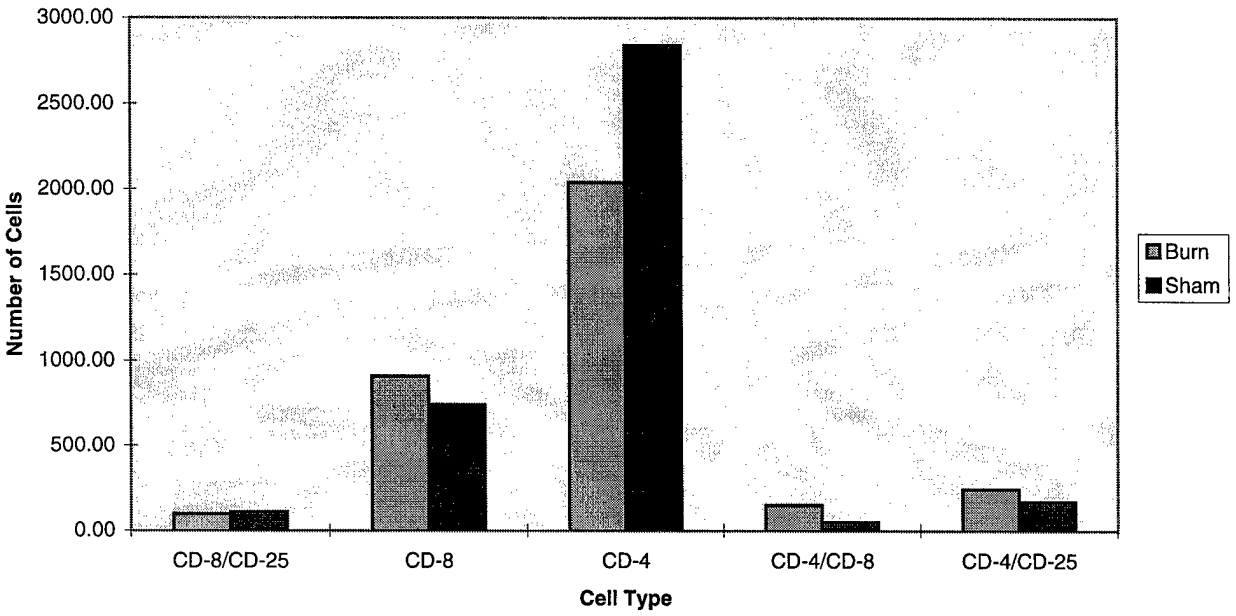
Figure 8: Flow Cytometry Post-Burn Day 3



	CD-8/CD-25	CD-8	CD-4	CD-4/CD-8	CD-4/CD-25
Burn	38.00	969.25	2023.50	205.00	196.50
Sham	46.00	941.50	2356.25	127.00	195.00
p-value	0.497	0.850	0.009	0.014	0.970

Figure 9

Figure 9: Flow Cytometry Post-Burn Day 14



	CD-8/CD-25	CD-8	CD-4	CD-4/CD-8	CD-4/CD-25
Burn	98.00	910.75	2038.75	150.50	244.50
Sham	110.50	742.25	2841.75	50.50	172.50
p-value	0.574	0.087	0.001	0.008	0.312

Figure 10

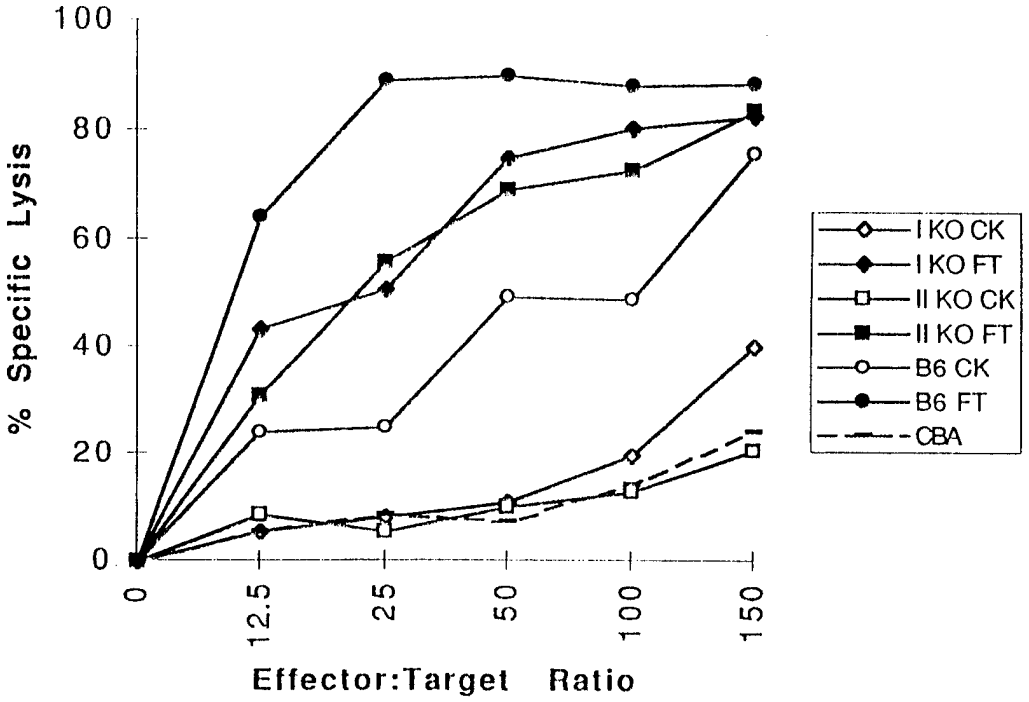


Fig 10 CTL alloreactivity of grafting groups at varied E:T ratios. CBA mice (n = 28) were flank grafted with class I knock-out cultured keratinocytes (I KO CK), class I knock-out full-thickness skin (I KO FT), class II knock-out keratinocytes (II KO CK), class II knock-out skin (II KO FT), normal allogeneic keratinocytes (B6 CK), normal allogeneic skin (B6 FT), and normal syngeneic keratinocytes (CBA). Three weeks after grafting, splenocytes were harvested from these groups and used as CTL effectors in a standard ^{51}Cr release assay. Radiolabeled, allogeneic EL-4 targets were mixed with effectors at varied ratios. Percent specific lysis of targets was determined via a γ-counter and was used as an index of CTL function. Mice primed by B6 FT, I KO FT, and II KO FT grafts generated the greatest CTL alloreactivity, followed by hosts primed with B6 CK grafts. Animals covered with CBA, I KO CK, and II KO CK grafts displayed negligible CTL alloreactivity.

Figure 11

Figure 11: IL-2 Levels

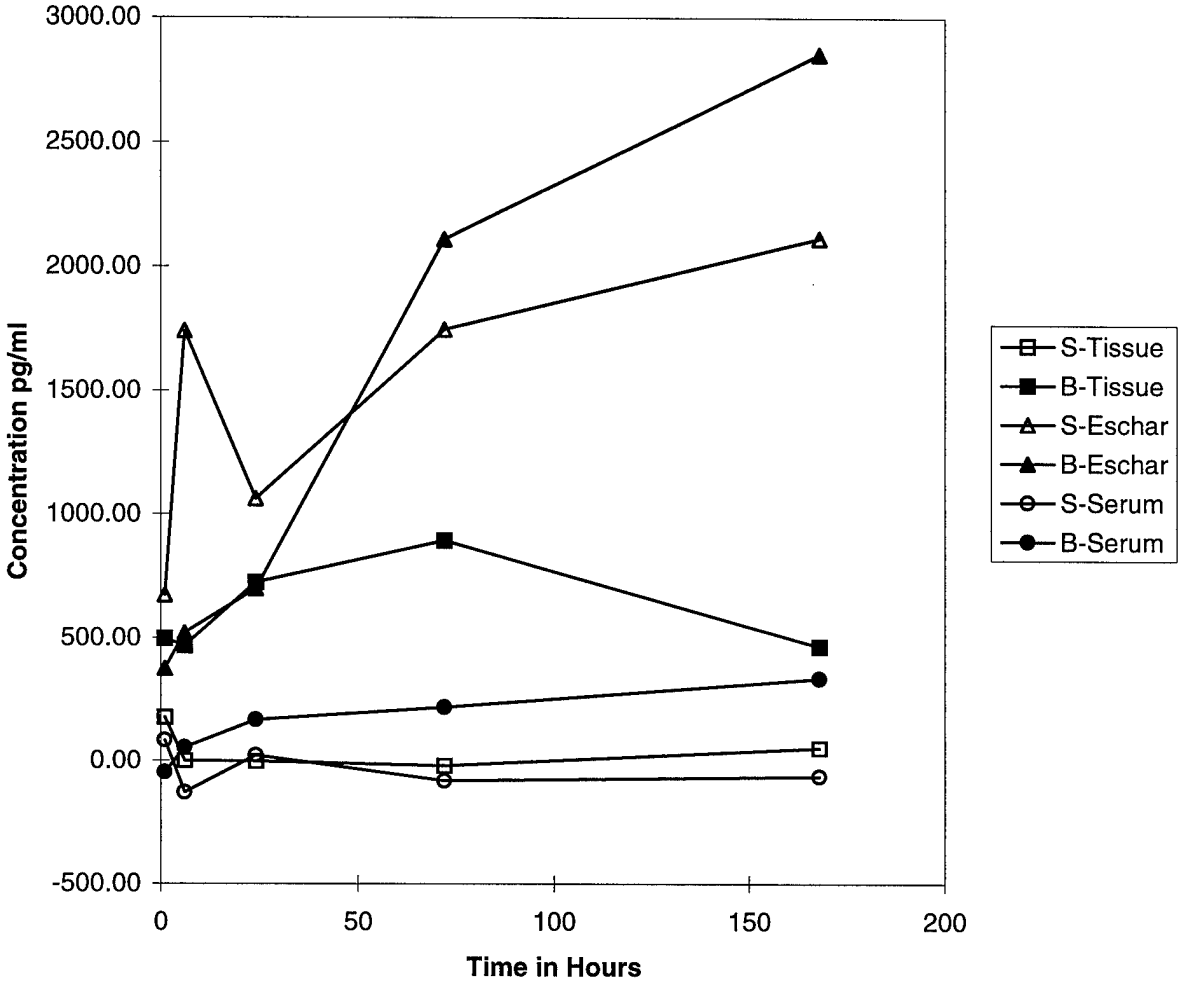


Figure 12

Figure 12: IL-4 Levels

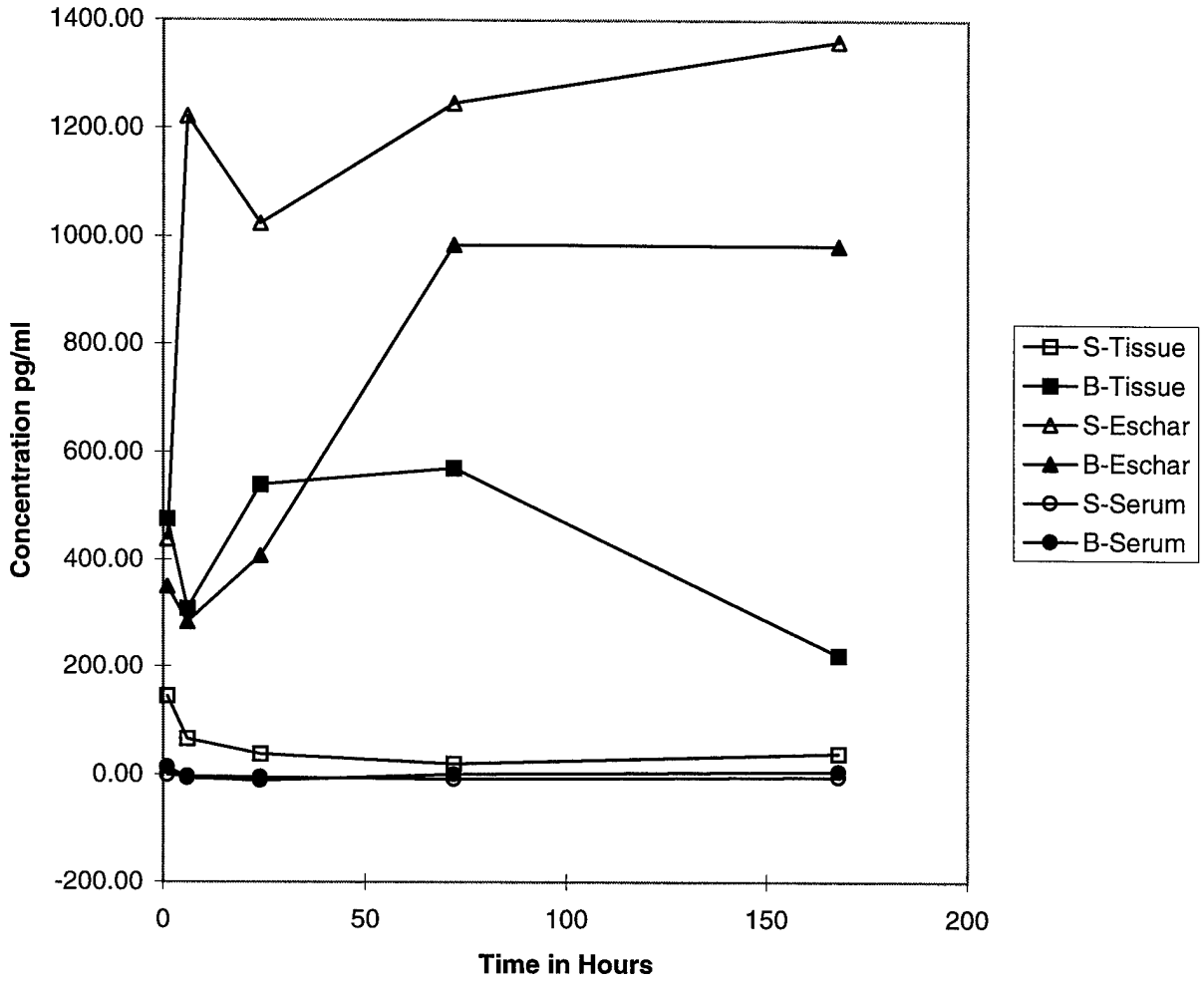


Figure 13

Figure 13: Interferon Gamma

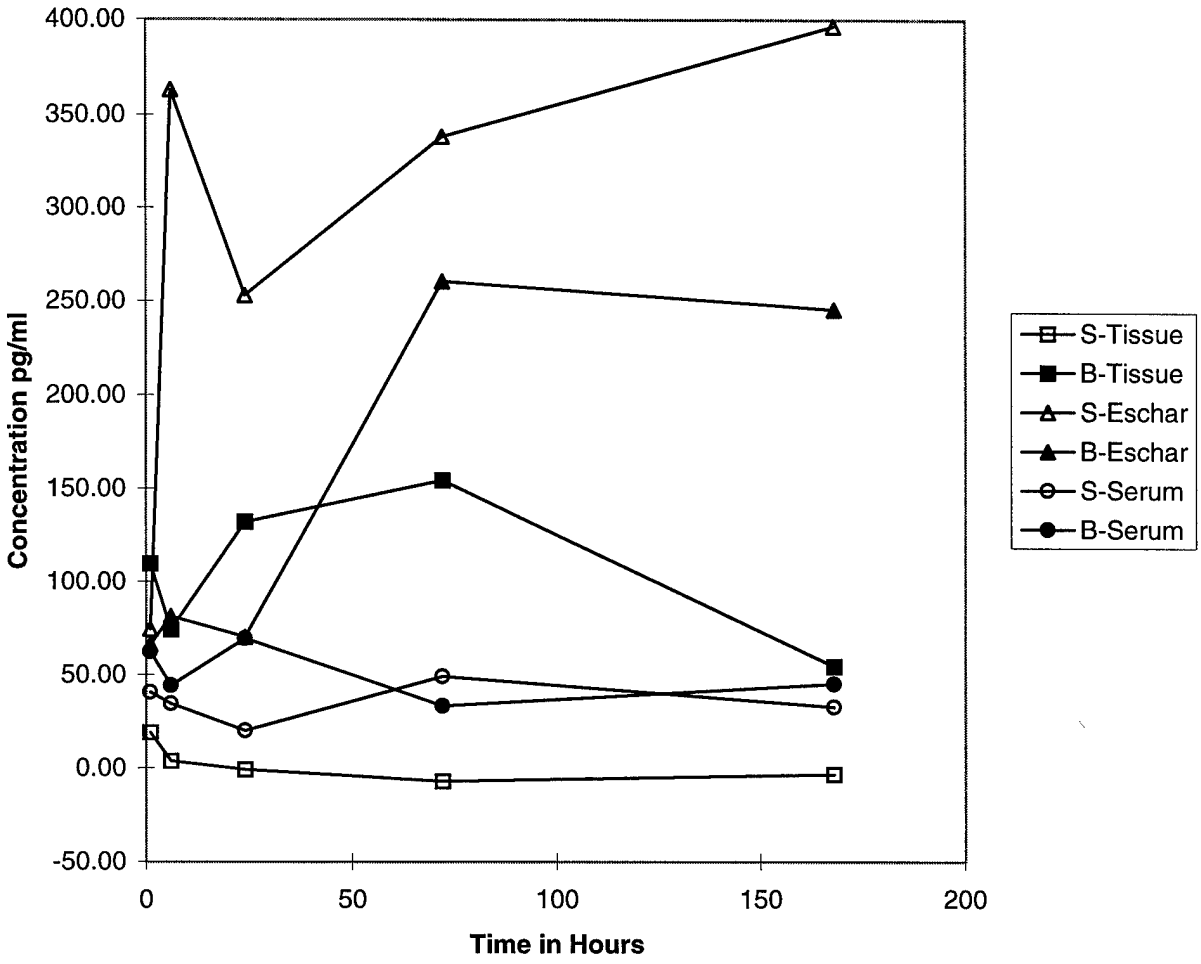


Figure 14

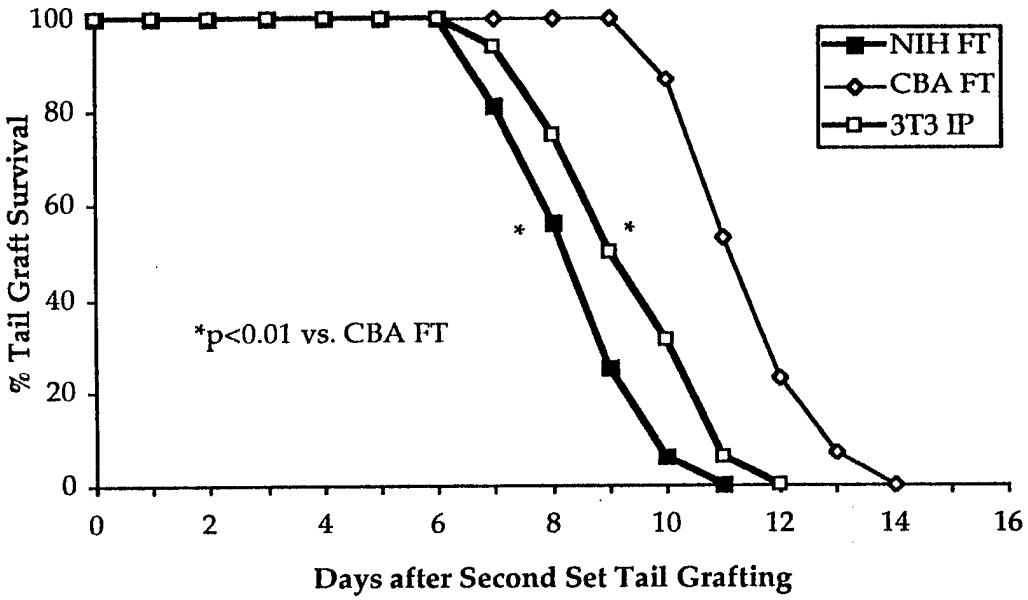


Fig 14 **The effect of antigen priming on second-set rejection. H-2^k hosts primed with H-2^q alloantigen from both NIH full-thickness allografts and intraperitoneal 3T3 fibroblasts reject second-set tail allografts more vigorously than hosts grafted initially with syngeneic CBA skin ($p < 0.01$). Abbreviations: FT, full-thickness; IP, intraperitoneal.**

Figure 15

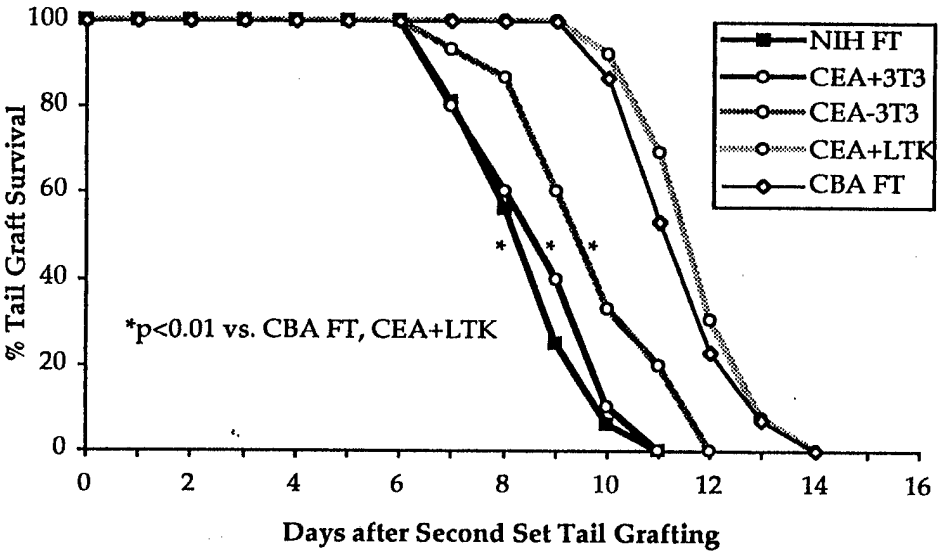


Fig 15 **The effect of an allogeneic feeder layer of second-set rejection. CEAs cultivated with an allogeneic H-2^q feeder layer (both removed and left intact) prime hosts for accelerated rejection of second-set tail allografts, compared to hosts receiving CEAs grown with a syngeneic H-2^k LTK feeder layer ($p < 0.01$). Abbreviations: FT, full-thickness; CEA, cultured epidermal autograft.**

APPENDIX 3: PAPERS

Paper 1: The 1995 MOYER AWARD. The Effect of Burn Injury on Allograft Rejection, Alloantigen Processing, and Cytotoxic T-Lymphocyte Sensitization

Paper 2: The Relationship Between Interferon-γ and Keratinocyte Alloantigen Expression After Burn Injury.

Paper 3: Allogeneic Keratinocytes Deficient in Class II Antigens Fail To Prime The Host For Accelerated Second-Set Rejection or Enhanced T Lymphocyte Cytotoxicity.

Paper 4: Allogeneic Fibroblasts Used to Grow Cultured Epidermal Autografts Persist in Vivo and Sensitize the Graft Recipient for Accelerated Second-Set Rejection.

THE 1995 MOYER AWARD
The Effect of Burn Injury on Allograft Rejection, Alloantigen Processing, and Cytotoxic T-Lymphocyte Sensitization

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Chapel Hill, North Carolina

Burn injury impairs cellular immunity, increases the risk of viral infection, and delays allograft rejection, but little is known about its effect on antigen processing and cytotoxic T-lymphocyte (CTL) function. This study examined the effect of burn injury on alloantigen sensitization with an in vivo model of second-set rejection and in vitro assays of CTL alloreactivity. Anesthetized CBA mice (n = 95) received a 0%, 20%, or 40% full-thickness contact burn that was partially excised 3 days later and covered with autograft or C57BL/6 allograft. Two weeks after the burn was inflicted, mice were challenged with second-set tail allografts, which were observed for rejection. Median graft survival times were compared by Wilcoxon rank and chi-squared analysis. Additional CBA mice (n = 24) underwent similar burn injury, excision, and grafting. Splenocytes were harvested 2 weeks later and were used as CTL effectors against radiolabeled targets. Dilution curves of target lysis were compared by analysis of variance. Forty percent burn injury prolonged unprimed allograft survival from 13 to 15 days ($p < 0.01$) but had a greater effect on primed allograft survival, which increased from 9 to 12.5 days ($p < 0.01$). Furthermore, a 40% burn eliminated the influence of priming, resulting in second-set graft survival similar to that of mice in an unburned, unprimed control group (12.5 vs. 13 days, NS). Whereas 20% burn injury did not inhibit CTL priming, a 40% burn profoundly impaired CTL function ($p < 0.001$), which recovered only after 6 days of in vitro allostimulation. Burn injury inhibits both alloantigen priming and the immunologic memory of CTLs as a function of burn size. This impairment in alloantigen processing helps to explain defects in cellular immunity and suggests a mechanism for prolonged allograft survival and decreased viral resistance after burn injury occurs. (J BURN CARE REHABIL 1995;16:573-80)

Through mechanisms not completely described or understood, burn injury produces defects in cellular immunity that ultimately result in host immunosuppression. Clinically, burn injury increases the risk of viral infection,[1] inhibits contact hypersensitivity,[2-4] and delays allograft rejection,[5-8] suggesting an impairment in antigen recognition, elimination, or both. Although burn injury has been shown to inhibit lymphocyte effector function[9-13] and to alter lymphocyte cytokine production,[14-17] little is known about the effect of burn injury on major histocompatibility complex (MHC)-restricted antigen processing, which involves the recruitment and activation of cytotoxic T lymphocytes (CTLs).

The ramifications of impaired CTL activity include allograft nonresponsiveness and decreased host resistance to opportunistic infections.[18,19] Although most postburn infections are bacterial in origin, the incidence and significance of viral infections may be underappreciated. Pediatric patients with major ther-

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Supported by U. S. Army grant DAMD 17-91-Z-1007, National Institutes of Health grant AI 20288, and the North Carolina Jaycee Burn Center.
The viewpoints expressed in this paper are those of the authors and do not necessarily represent those of the U. S. Army or the Department of Defense.
Presented at the Twenty-seventh Annual Meeting of the American Burn Association, Albuquerque, N.M., April 19-22, 1995.
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30/1/66107

mal injury appear to be at increased risk for cytomegalovirus infection, which manifests itself as hepatitis and persistent fever.[1] Another consequence of postburn immunosuppression is prolonged allograft survival,[5-8] which may also be related to defects in antigen processing and CTL function. The prolonged survival of cadaveric, full-thickness skin improves patient outcome by permitting more aggressive wound excision and enabling complete wound closure. However, such functional wound coverage is only temporary, because allogeneic epidermal cells are eventually rejected upon restoration of host immunocompetence. Although not yet achieved, the induction of permanent allograft tolerance remains a challenging but desirable goal in burn wound management.

The purpose of this study was to characterize the effect of burn injury on alloantigen processing. Specifically, we were interested in studying how thermal injury affects alloantigen sensitization. First described by Gibson and Medawar[20] in 1943 as second-set rejection, sensitization (also known as priming) involves a phenomenon in which animals previously exposed to a specific antigen will mount a more vigorous rejection response when reexposed to that antigen. With an in vivo model of second-set rejection, we investigated the effect of burn injury on naive and primed allograft rejection. Additionally, in vitro CTL assays were used to elucidate the influence of burn injury on MHC-restricted, target-specific cytotoxicity. Together, the results of these studies suggest that burn injury inhibits antigen processing as a function of burn size and that primed allograft rejection is impaired to a greater extent than naive rejection.

MATERIAL AND METHODS

Experimental Design. To determine the effect of burn injury on alloantigen processing, we used an in vivo model of second-set rejection and in vitro assays of CTL function to study the influence of burn size on alloantigen sensitization. In the first series of experiments 95 CBA mice were randomized to receive a 0%, 20%, or 40% total body surface area (TBSA) burn, which was partially excised 3 days later and covered with either CBA autograft ($n = 45$) or C57BL/6 allograft ($n = 50$). Two weeks after grafting was performed, the animals were then challenged with second-set tail allografts, which were observed for rejection. In the second series of experiments 24 CBA mice received a 0%, 20%, or 40% TBSA burn, which was partially excised 3 days later and covered with C57BL/6 allograft. Two weeks after priming was performed, splenocytes were harvested, stimulated with alloantigen, and used in subsequent CTL assays. Splenocytes were also collected from a fourth group of unburned, autografted, CBA mice. This group served as a negative control group, representing the baseline activity of unprimed CTLs against allogeneic targets.

Animal Protocols. Female CBA/J mice (4 to 6 weeks old, 15 to 20 gm) ($H\text{-}2^k$) (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were used as graft recipients and autograft donors in both experiments. Allogeneic splenocytes needed as stimulators in the CTL assays were obtained from age-, weight-, and sex-matched C57BL/6 mice ($H\text{-}2^b$) (Charles Rivers Laboratories, Wilmington, Mass.), which were also used as allograft donors in both experiments. Animal protocols conformed to National Institutes of Health guidelines and were approved by the University of North Carolina Committee on Animal Research.

Burn Injury Model. After general anesthesia was induced with methoxyflurane (Pitman-Moore, Washington Crossing, N. J.), circumferentially clipped mice received a full-thickness flank/back contact burn via a 10-second application of a 65gm brass rod previously heated to 100° C. Animals were then resuscitated with intraperitoneal lactated Ringer's solution (0.1 ml/gm body weight), given subcutaneous morphine sulfate (3 μg/gm body weight), and returned to individual cages to feed ad libitum.

Concerning the contact burn, one application represents 10% of the animal's TBSA; four separate applications are necessary to create a 40% TBSA burn. The sham group receiving the 0% TBSA burn had all of the previously mentioned interventions with the exception of the rod application. The contact burn produces a wound of consistent depth and border, which allows for precise excision and grafting. Predictable mortality rates in unexcised mice include 0% for the sham burn, 10% for the 20% TBSA burn, and 40% for the 40% burn. In this burn model wound infection rarely occurs, and mortality is typically the result of generalized sepsis, which occurs 3 to 10 days after injury. Autopsy reveals intestinal dilatation and edema consistent with ileus and partial obstruction.

Wound Excision and Primary Flank Grafting. Three days after burn injury was inflicted, flank wounds were partially excised, leaving a significant amount of eschar intact to maintain host immunosuppression. Full-thickness autografts or allografts previously debrided of adipose and washed in phos-

Table 1. Effect of burn injury and priming on the survival of second-set tail allografts

	Primed			Unprimed		
	Primary flank graft: allograft			Primary flank graft: autograft		
	0% Burn	20% Burn	40% Burn	0% Burn	20% Burn	40% Burn
MST, days	9	10	12.5	13	14	15
MST, range	7-11	8-12	10-16	10-15	12-17	13-18
No.	18	16	16	15	17	13

Mice receiving primary flank allografts are defined as primed, whereas mice receiving primary flank autografts are defined as unprimed. *MST*, Median survival time of secondary tail allografts.

phate-buffered saline solution were placed directly onto the exposed musculoskeletal facsia, were secured with skin staples, and were covered with a circumferential adhesive bandage, which was removed after 1 week. This sequence of burn injury, excision, and grafting was used to approximate the clinical practice of early excision and grafting.

Second-Set Tail Grafting. To determine the effect of thermal injury on host sensitization, mice were challenged with alloantigen 2 weeks after burn injury, excision, and grafting were performed. Anesthetized mice underwent second-set tail grafting via a previously described method[21] in which a 10 mm full-thickness skin allograft (C57BL/6) was placed on the dorsal tail surface and compared with distally placed 10 mm full-thickness autograft (CBA) serving as an internal control sample. Tail grafts were protected from mechanical disruption and organic debris by specifically designed cylindric glass tubes, which were removed 3 days later. Two independent observers assessed tail graft viability daily. Evidence for rejection was based on the objective criteria of graft color, scale integrity, and hair orientation. Median survival time of second-set tail allografts was compared among the six groups and assessed for statistical significance.

CTL Alloreactivity. To determine the effect of burn injury on MHC-restricted CTL reactivity and sensitization, we studied CTL function in CBA mice receiving burn injury, partial wound excision, and flank allografting. Two weeks after sensitization with alloantigen was performed, splenocytes were harvested, washed in culture media (Roswell Park Memorial Institute 1640 media with 10% fetal bovine serum, 0.1% penicillin/streptomycin, and 5×10^{-5} M 2-ME, all obtained from the Lineberger Cancer Center, Chapel Hill, N. C.), and used in subsequent CTL assays. Graft recipient splenocytes (2×10^6 cells/ml, CBA mice, H-2^k) were cocultured with growth-arrested (2000 rads) donor splenocytes (2×10^6 cells/ml, C57BL/6, H-2^b) in standard incubator conditions of 37° C and 5% CO_2.

After 0 to 7 days of in vitro stimulation, CTL alloreactivity was determined by collecting effector lymphocytes and testing them on both allogeneic positive targets (EL-4 murine lymphoma cells, H-2^b, ATCC TIB 39) and MHC-identical negative targets (LTK$^-$, H-2^k, ATCC CCL 1.3). After having been preloaded with 100 μCi of $Na_2{}^{51}CrO_4$ (ICN Biomedicals Inc., Irvine, Calif.) for 30 minutes, targets were placed in round-bottom 96 well plates (1×10^6 cells/well) and mixed with CTL effectors at ratios of 1:12.5, 1:25, 1:50, 1:100, and 1:150. After a 4-hour, 37° C incubation in 150 μl Roswell Park Memorial Institute media 1640 with 5% fetal bovine serum, target lysis was determined by measuring the release of $Na_2{}^{51}CrO_4$ into the media. Collected supernatants were assessed for radioactivity (expressed as mean cpm) by a γ-counter. Each condition was tested in duplicate. Specific target killing was determined by the following formula: % specific lysis = $([cpm_{sample} - cpm_{spontaneous}]/[cpm_{maximum} - cpm_{spontaneous}]) \times 100$, where $cpm_{spontaneous}$ represents spontaneous target release, and $cpm_{maximum}$ represents the radioactivity of targets lysed by 5% Triton X-100 (Sigma, St. Louis, Mo.) Average spontaneous target release was less than 10% of the potential maximum release. A total of 12 assays were performed after 0, 3, 4, 5, 6, and 7 days of stimulation against positive and negative targets. This experiment was repeated and yielded similar results.

Statistics. Median survival times of second-set tail allografts were compared among groups by Wilcoxon rank and chi-squared analysis. CTL alloreactivity as signified by percent specific lysis was compared among groups by two-way analysis of variance with replication. Statistical significance was defined for differences where $p < 0.05$.

RESULTS

Second-Set Rejection. To characterize the effect of thermal injury on alloantigen processing, we used a model of second-set rejection in which previously burned mice underwent wound excision and flank grafting followed 2 weeks later by secondary tail allografting. We hypothesized that previous exposure to alloantigen would result in a more vigorous rejection response when mice were challenged with second-set allografts. Furthermore we hypothesized that burn injury would impair both primed and unprimed rejection of tail allografts, as indicated by prolonged survival of the second-set tail grafts.

Median tail allograft survival times and range and group size are listed in Table 1. Survival curves depicting graft survival as a function of time can be found in Fig. 1. As expected, unburned mice primed with alloantigen rejected second-set allografts more rapidly than did the unburned, autografted group (9 vs. 13 days, respectively; $p < 0.001$). Burn injury increased tail allograft survival in both primed and unprimed groups. Although burn injury prolonged unprimed graft survival in the 20% TBSA burn group (from 13 to 14 days, $p < 0.01$) and the 40% burn group (from 13 to 15 days, $p < 0.001$), the difference between these two groups was not significant. In the primed group a 20% burn increased tail allograft survival from 9 to 10 days ($p < 0.05$), and a 40% burn prolonged allograft survival from 9 to 12.5 days ($p < 0.001$). The difference between these primed groups (20% vs. 40% burn) was statistically significant ($p < 0.01$). Although burn injury impaired allograft rejection in both the primed and unprimed groups, burn injury had a greater effect in the primed group, suggesting a specific defect in alloantigen sensitization. Furthermore a 40% burn eliminated the effect of priming when compared with allograft rejection in unprimed, unburned mice (12.5 vs. 13 days, NS).

CTL Alloreactivity. After using this in vivo model of rejection to study the effect of burn injury on antigen processing, we then asked whether defects in CTL activity might account for this observation of impaired alloantigen priming. Splenocytes from allografted mice who had received a 0%, 20%, or 40% burn and from autografted mice who had received a 0% burn were cocultured with growth-arrested allogeneic stimulators and used as effectors in subsequent CTL assays. Dilution curves representing specific lysis of positive and negative targets are depicted in Figs. 2 through 5.

CTL alloreactivity was not observed until the third day of in vitro stimulation. Unburned, primed mice and primed mice receiving a 20% TBSA burn injury displayed the greatest CTL activity against positive targets (Fig. 2, $p < 0.001$). CTLs from unburned, unprimed mice and from primed mice with the 40% burn demonstrated no significant activity compared with the nonspecific lysis of negative targets. After 4 days of in vitro stimulation, CTL activity improved in all of the primed groups, but profound suppression of CTL activity by a 40% burn was still observed (Fig. 3, $p < 0.001$). Whereas the 20% primed group had slightly decreased CTL function compared with the 0% primed group, the 40% primed group demonstrated insignificantly improved CTL function compared with the unprimed, unburned group.

CTL activity in the unburned, unprimed control group improved dramatically after 5 days of stimulation (Fig. 4). However, depressed CTL alloreactivity in the 40% burn group persisted and did not fully recover until the final day of the assay (Fig. 5). After 6 days of simulation primed CTLs from all three burn groups demonstrated greater activity than unprimed CTLs except at an effector/target ratio of 1 : 100. This complete recovery of impaired CTL function after burn suggests that defects in antigen priming, although significant, are not permanent and can be reversed by in vitro stimulation with alloantigen.

DISCUSSION

In this series of experiments we provide evidence that thermal injury produces identifiable defects in alloantigen processing. Specifically, burn injury inhibits both primed and unprimed allograft rejection, with the degree of immune dysfunction dependent on the size of the burn wound. Furthermore alloantigen sensitization appears to be more impaired than naive alloantigen elimination in our model of second-set rejection. These in vivo findings correlate with in vitro studies of CTL alloreactivity after burn injury. Whereas a 20% TBSA burn had minimal effect on primed CTL target cytotoxicity, a 40% burn dramatically mitigated CTL sensitization. Although 40% burn injury negated the immunologic memory of CTLs, target-specific cytotoxicity fully recovered but only after 6 days of in vitro stimulation with alloantigen.

Immunosuppression after thermal injury has been extensively studied, but the full range of defects in cellular immunity has yet to be described. Assays of cellular immune function after burn reveal a decreased lymphocyte proliferation in response to mitogens,[14,22] decreased lymphocyte production of in-

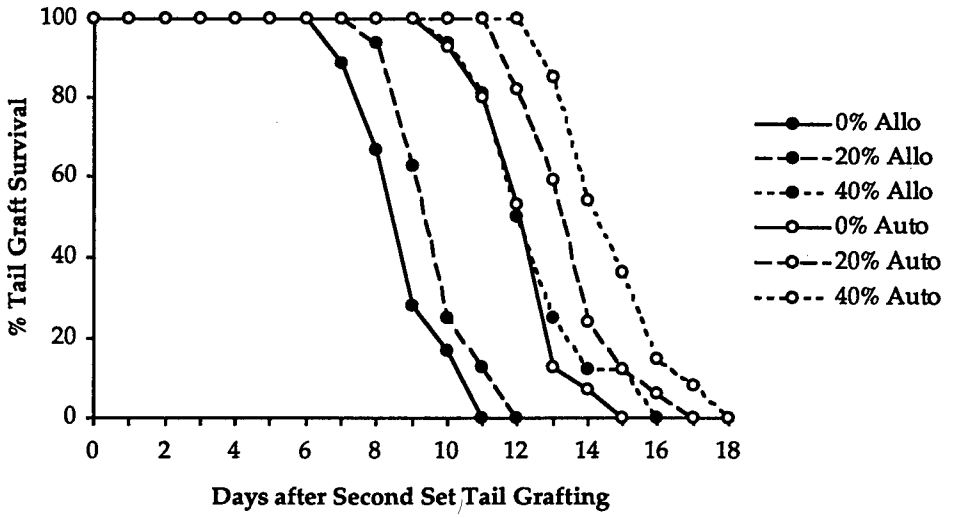

Figure 1. Survival curves of secondary tail allografts depicting effect of thermal injury and priming on second-set rejection. Burn injury significantly prolonged second-set tail graft survival in both the primed *(Allo)* and unprimed *(Auto)* groups ($p < 0.05$). Sensitized rejection was impaired to a greater extent than naive rejection.

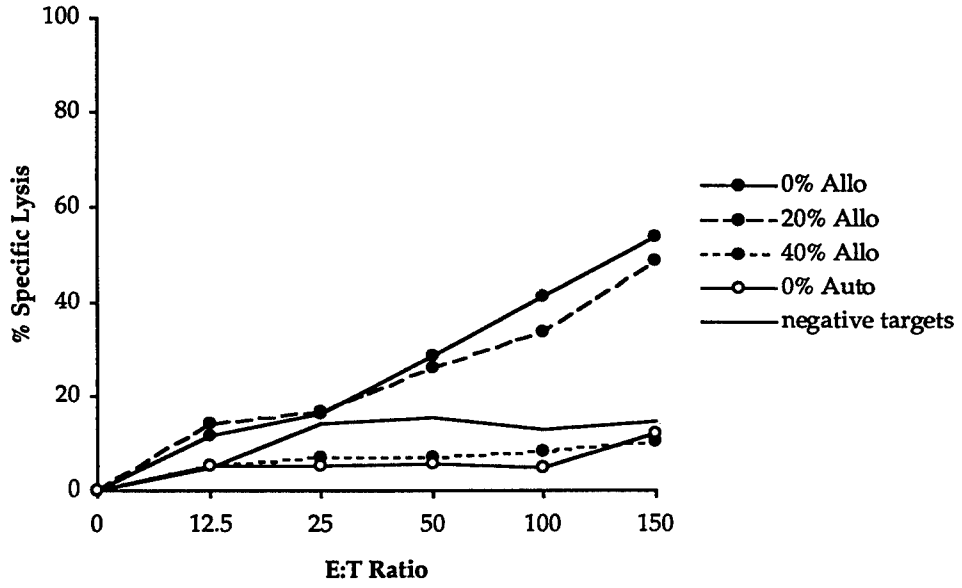

Figure 2. Effect of burn injury on CTL alloreactivity 3 days after in vitro stimulation.

terleukin-2,[14,15] and decreased lymphocyte effector function.[9-13] Furthermore fluorescence-activated cell sorting demonstrates decreased numbers of T helper and T cytotoxic lymphocytes and the emergence of suppressor lymphocytes in both human and animal models of burn injury.[23-25]

This disruption of cellular immunity has significant clinical consequences, increasing the risk for infectious complications, which remain the leading cause of postresuscitation deaths after burn injury.[26] Although gram-negative sepsis accounts for most postburn infections,[27] the incidence of viral, fungal, and other opportunistic infections is occurring with increasing frequency and may not be fully appreciated.[28] In a prospective case series Linnemann and MacMillan[1] reported that cytomegalovirus infection

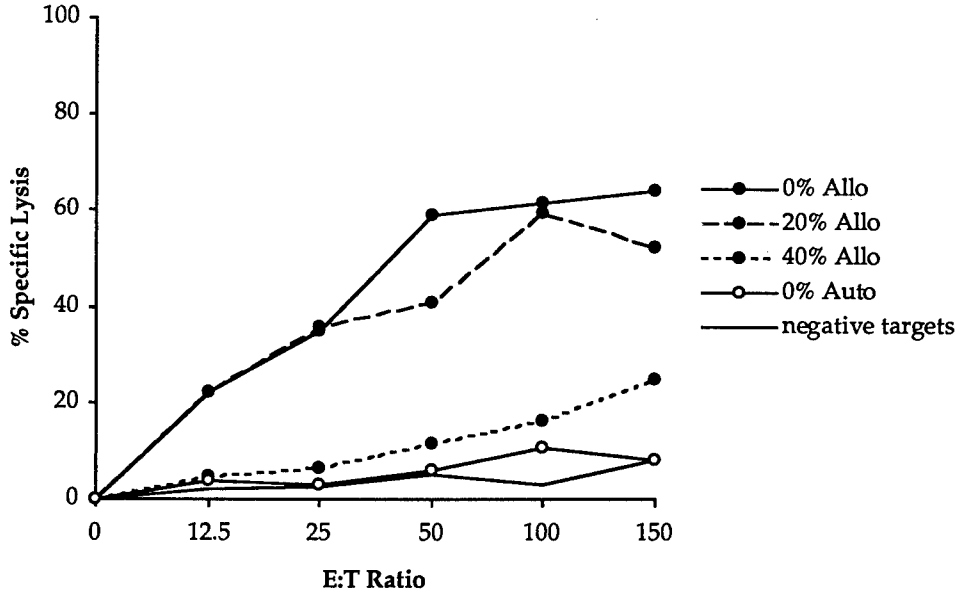

Figure 3. Effect of burn injury on CTL alloreactivity 4 days after in vitro stimulation.

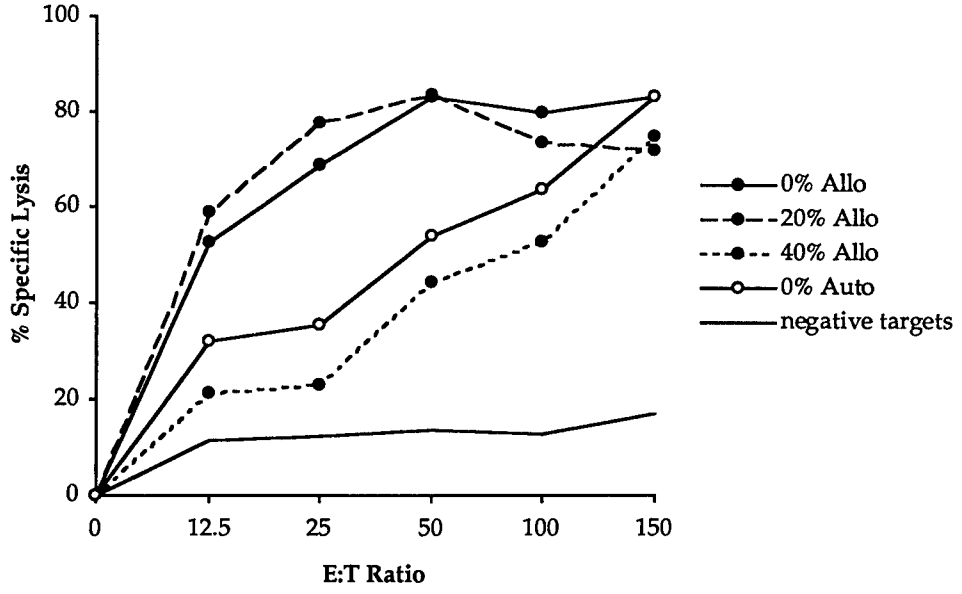

Figure 4. Effect of burn injury on CTL alloreactivity 5 days after in vitro stimulation.

occurred in 33% of pediatric patients with burns and that herpes simplex virus infection occurred in 25%. Additionally, those patients with larger burns were more likely to have antiviral antibodies and have positive viral cultures. This susceptibility to viral infection suggests that burn injury impairs the effector function of CTLs, the component of cellular immunity responsible for MHC-directed elimination or clearance of viral antigen.

Impaired antigen processing after burn injury occurs is implicated by defects in contact hypersensitivity, which is a nonspecific but quantitative indicator of cell-mediated immunity. Hansbrough et al.[2,3] have shown that a 20% burn injury can significantly reduce contact hypersensitivity, as measured by ear swelling, in animals that were sensitized with and reexposed to dinitrofluorobenzene. Early wound excision restores contact hypersensitivity in the burned host,[2] whereas transfer of the burn eschar to unburned hosts also transfers these defects in antigen processing.[3]

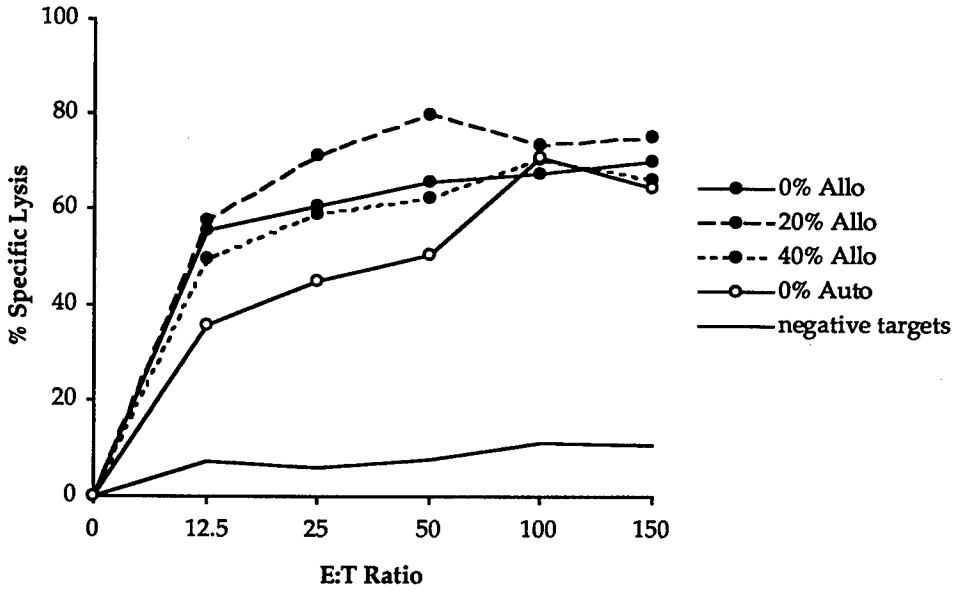

Figure 5. Effect of burn injury on CTL alloreactivity 6 days after in vitro stimulation.

Other evidence for impaired antigen processing after burn occurs comes from the observation that burn injury mitigates the host-versus-graft response. Cetinkale et al.[4] noted that burn injury decreases the alloreactive response when a host is challenged with alloantigen, as measured by the popliteal lymph node assay. Furthermore prolonged allograft survival after burn occurs is well documented[5-8] and has been used to permit early wound excision of the massively burned patient. Markley et al.[6] reported that burn injury and septic and hyperosmolar shock produced considerable immune dysfunction such that allogeneic skin grafted 3 weeks after injury had prolonged survival. Additionally, second-set allografts placed 70 days after burn injury also had prolonged survival, suggesting a long-lasting defect in alloantigen sensitization. Although our experimental model involved much earlier excision and grafting, these results are consistent with our findings that burn injury impairs primed and unprimed allograft rejection.

The mechanism for delayed allograft rejection may be related to postburn defects in CTL function, which is a critical effector component of allograft rejection. Previous work by Markley et al.[9] demonstrates that a 66% TBSA burn injury inhibits the effector function of CTLs from BALB/c mice presensitized with an intraperitoneal injection of allogeneic EL-4 cells. This impaired sensitization was present when burn injury occurred between 7 days before and 13 days after exposure to alloantigen. Furthermore burn injury appeared to inhibit the afferent side of alloantigen sensitization, in which EL-4 cells from burned C57BL/6N donors failed to prime BALB/c hosts for target-specific CTL cytotoxicity.[10] In our experiment mice were primed instead with alloantigen from skin allografts, and we observed that a 40% burn injury was required to inhibit primed CTL alloreactivity.

Successful long-term coverage with allografts remains an elusive goal in burn wound management. Although allograft rejection can be delayed by the use of cyclosporin[29-31] and azathioprine,[32] by allograft irradiation or treatment with glucocorticoids[33] or after bone marrow transplantation[8] skin allografts used for burn wound coverage are ultimately rejected upon restoration of host immunocompetence. Understanding how burn injury affects alloantigen processing, however, may someday permit the manipulation of host, graft, or both to effect the permanent survival of allografts in burn wound coverage.

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Reprinted from: ANNALS OF SURGERY, Vol. 222, No. 3, September 1995

The Relationship Between Interferon-γ and Keratinocyte Alloantigen Expression After Burn Injury

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ANNALS OF SURGERY
Vol. 222, No. 3, 384–393

The Relationship Between Interferon-γ and Keratinocyte Alloantigen Expression After Burn Injury

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Background

Cultured keratinocyte (CK) and cadaveric skin allografts have prolonged survival in patients with massive thermal injury. It is unclear if this delayed rejection is due to impaired host responsiveness or decreased graft immunogenicity. Although burn injury has been shown to decrease parameters of allograft response, no studies have examined the effect of burn injury on alloantigen expression. This study investigated the effect of burn size on class II antigen expression in CK allografts as well as on tissue levels of interferon-γ (IFN-γ), the principle regulator of alloantigen expression.

Methods

Anesthetized CBA mice (n = 64) received a 0%, 20% partial-thickness (PT), 20% full-thickness (FT), or 40% FT contact burn. Forty-eight hours later, wounds were partially excised and covered with CK allografts from C57BL/6 donors. Five days after burn injury, grafts were analyzed for donor-specific class II antigen. Protein expression was determined by Western immunoblotting and quantified with video densitometry. Wound, serum, and unburned skin levels of IFN-γ were determined by enzyme-linked immunosorbent assay. Groups were compared by Fisher's analysis of variance.

Results

As burn size increased, class II antigen expression decreased ($p < 0.001$). This corresponded with decreased wound and skin levels of IFN-γ after 40% burn ($p < 0.05$); however, wound IFN-γ was significantly elevated after 20% PT and FT burns ($p < 0.01$). Serum IFN-γ increased as burn size increased ($p < 0.01$).

Conclusions

Burn injury decreases the antigenicity of CK allografts, which partly explains delayed allograft rejection after burn injury. Although wound IFN-γ increases after minor thermal injury, the profound decrease in wound and skin IFN-γ after a major burn corresponds with diminished class II antigen expression. The decreased availability of IFN-γ after major thermal injury provides a mechanism for limited allograft tolerance.

Cultured keratinocyte (CK) allografts have been proposed as a skin replacement in patients with massive thermal injury.[1-3] These patients represent a significant challenge to the burn surgeon, because early excision and permanent closure of the burn wound may not be possible. The lack of donor sites limits the quality and quantity of autologous skin that can be harvested for wound coverage. Full-thickness allografts can be used as a temporary biologic dressing, but these grafts are eventually rejected unless the host remains immunosuppressed.[4]

Cultured keratinocyte allografts are particularly attractive due to their inherently limited immunogenicity. These epidermal sheets do not contain passenger leukocytes, because highly antigenic Langerhans' cells found in skin do not persist in tissue culture.[5] Furthermore, keratinocytes do not constitutively express major histocompatibility complex (MHC) class II antigen,[6] which is critically involved in allograft recognition and rejection. Additionally, CK allografts fail to elicit cytotoxic antibody once grafted and do not generate a mixed-lymphocyte response.[7]

Recent work, though, suggests that CK allografts are more immunogenic than previously believed. Cultured keratinocyte allografts prime the unburned host for accelerated second-set rejection and activate cytotoxic T lymphocytes.[8] Additionally, keratinocytes will express MHC class II antigen when exposed to interferon-gamma (IFN-γ) *in vitro*[9] or *in vivo*.[10] Repeated application of these grafts in the immunocompetent host may create a chronic inflammatory state counterproductive to wound healing. Although CK allografts are not acutely rejected, the long-term survival of these grafts is unknown, because they are gradually replaced by host keratinocytes.[11]

The immunosuppression of thermal injury, however, may decrease the immunogenicity of CK allografts and improve their function as a permanent skin replacement in burn wound coverage. We have recently demonstrated that burn injury selectively impairs host sensitization to CK allografts compared with full-thickness allografts.[12,13] Although this decreased host responsiveness may be due to defects in cell-mediated immunity, perhaps burn injury, with its altered cytokine profiles, affects the antigenicity of the actual grafts. The purpose of this study was to determine if burn injury inhibits the induction and expression of MHC class II antigens in CK allografts. Furthermore, we will investigate the effect of burn injury on tissue levels of IFN-γ, the principle cytokine that regulates expression of MHC class II antigen.

Presented at the 115th Annual Meeting of the American Surgical Association, April 6–8, 1995, Chicago, Illinois.

Supported by U.S. Army grant DAMD 17-91-Z-1007 and the North Carolina Jaycee Burn Center.

The viewpoints expressed in this paper are those of the authors and do not necessarily represent those of the U.S. Army or the Department of Defense.

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Accepted for publication April 10, 1995.

MATERIALS AND METHODS

Experimental Design

This study examined the effect of burn injury on MHC class II antigen expression in CK allografts as well as the effect of burn injury on the production of IFN-γ. In the first experiment, CBA mice (n = 36) received one of four burn wounds: 0% total body surface area, 20% partial-thickness (PT), 20% full-thickness (FT), or 40% FT. Forty-eight hours after burn, wounds were partially excised and covered with CK allografts, which were biopsied 3 days later. Specimens were then analyzed for donor-specific MHC class II antigen by Western immunoblotting.

In the second experiment, CBA mice (n = 28) received 0%, 20% PT, 20% FT, and 40% FT burn injuries, which again were partially excised and covered with CK allografts derived from C57BL/6 mice. Three days after grafting (5 days after burn injury), serum, wound, and skin samples were collected and processed to determine levels of IFN-γ. An enzyme-linked immunosorbent assay (ELISA) was used to measure levels of this cytokine.

Animal Protocols

Four- to six-week-old female CBA/J ($H\text{-}2^k$) mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 15 to 20 g were used as graft recipients in both the MHC class II antigen and IFN-γ experiments. Graft donors were age/weight-matched female C57BL/6 ($H\text{-}2^b$) mice (Charles Rivers Laboratories, Wilmington, MA). All protocols were approved by the University of North Carolina Committee on Animal Research and were in accordance with National Institutes of Health guidelines.

Burn Injury

Animals were anesthetized with methoxyflurane (Pitman-Moore, Washington Crossing, NJ) and circumferentially clipped. Burn injury was accomplished by the application of a 65-g copper rod, previously heated to 100 C., to the animal's back and flank for 10 seconds in FT wounds and 5 seconds in PT wounds. Each application represented 10% of the animal's total body surface area, with four applications necessary to produce a 40% burn. The contact burn described in this model creates

an injury of predictable depth and border, permitting precise wound excision and grafting.

All mice were then resuscitated with intraperitoneal lactated Ringer's solution (0.1 mL/g body weight) and were given subcutaneous morphine sulfate (3μg/g body weight) for postburn pain control. Animals were returned to individual cages to feed *ad libitum*. Mice receiving the 0% sham burn underwent all of these interventions, with the exception of the application of the copper rod.

Keratinocyte Cultures

Approximately 3 weeks before burn injury, keratinocyte cultures were prepared using methods modified from the technique described by Rheinwald and Green.[14] All tissue culture media and additives were obtained from the Lineberger Cancer Center (Chapel Hill, NC), except where noted.

Tail skins were obtained from C57BL/6 donors, washed with 70% ethanol, and stored overnight in Dulbecco's modified Eagle Medium (DMEM) supplemented with 0.1% penicillin/streptomycin. The skin was then incubated in 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) for 2 hours at 37 C. and in 5% carbon dioxide. The epidermis was separated from the dermis and vortexed to create a single cell suspension. These keratinocytes were co-cultured at a 2:1 ratio with a growth-arrested feeder layer of murine connective tissue cells (LTK, H-2^k) previously exposed to mitomycin C (Sigma Chemical Co.) for 45 minutes. Cells were grown in a solution of DMEM and Ham's F-12, which contained 5% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT), cholera enterotoxin (Schwartz Mann/ICN Biochemical Inc., Costa Mesa, CA), hydrocortisone 0.4 mg/mL, transferrin 5.0 mg/mL, insulin 5.0 mg/mL (all from Sigma Chemical Co.), and amphotericin 5.0 mg/mL (E.R. Squibb and Sons Inc., Princeton, NJ). Media were changed every 2 to 3 days, and epidermal growth factor 10.0 ng/mL (Collaborative Research Inc., Bedford, MA) was added after the first media change. Seven to 10 days after plating, LTK cells were removed by differential trypsinization, and keratinocytes were grown to confluence.

Grafting Procedure

Forty-eight hours after burn injury, wounds were partially excised and grafted with CK allografts. This time period was selected to approximate the clinical course of early excision and grafting in humans. Furthermore, most of the burn wound was left intact to ensure continued host immunosuppression. Burn wound depth was confirmed by histologic study of biopsies obtained at the time of excision.

On reaching confluence, CK allografts were released from culture dishes with the enzyme dispase (Boehringer Mannheim, Germany), placed on petroleum gauze "basal side up," and stored in the incubator until grafting. C57BL/6 allografts were then grafted onto the left flank of CBA recipients after excision of the wound to musculoskeletal fascia. Gauze-backed grafts were tucked under the surrounding wound edge "basal side down" and covered with a Vigilon hydrophilic dressing (C.R. Bond Inc., Berkeley Heights, NJ), which was secured with a stretch fabric bandage and skin staples.

Wound Assessment

On postburn day 5, 3 days after grafting, wounds were inspected, photographed, and biopsied with a 3-mm punch probe. Structural persistence of CK allografts was determined by fixing the tissue in 10% formalin; biopsies were later dehydrated, infiltrated with methyl-methacrylate, embedded in a gelatin capsule, sectioned on a GB4A microtome, and stained with methylene-blue acid-fuschin for histochemical analysis.

Western Immunoblotting

Donor-specific MHC class II antigen expression in CK allografts was determined by Western immunoblotting. Wounds were biopsied 3 days after grafting to correspond with maximal *in vivo* class II expression, which we have previously observed and reported.[8] Protein lysates were obtained by solubilizing biopsies in 200 μL of 0.001 M Tris-HCl, pH 7.4, 0.150 M NaCl, 0.5% NP-40, 1-mM phenylmethylsulfonyl fluoride (PMSF). Specimens were then vortexed, incubated on ice for 15 minutes, centrifuged for 15 minutes at 4 C., stored at −80 C., and standardized for protein concentration before immunoblotting. Polyacrylamide 12% sodium dodecyl sulfate gels were created with a Bio-Rad Model 360 vertical slab minicell and a Model 361 casting chamber (Bio-Rad Laboratories, Hercules, CA). Detergent-extracted specimens and molecular-weight markers were run on one-dimensional gels and transferred to nitrocellulose, which was then blocked with 1% bovine serum albumin. The blots were incubated with KL295, a murine monoclonal antibody that recognizes the denatured 30-kD α chain of H-2^b (but not H-2^k) MHC class II antigen. After primary antibody exposure, blots were incubated with goat anti-mouse antibody labeled with alkaline phosphatase to mark any murine immunoglobulin present on the Western blot.

Video Densitometry

Expression of donor MHC class II antigen was quantified with video densitometry. Western immunoblots were scanned with Macintosh video recording equipment (Apple Computer, Cupertino, CA), stored as video images in Power Point software (Microsoft, Redmond, WA), and analyzed with Gel Capture software (National Institutes of Health, Bethesda, MD). The amount of MHC class II antigen present was objectively determined by measuring the pixel density for each protein band. In an effort to minimize gel-to-gel variability, we compared antigen expression by calculating band strength as a proportion of the protein standard [(absolute pixel density of protein band)/(background density of the protein marker lane)].

Collection of Tissue Samples for Interferon-γ Levels

Three days after grafting (5 days after burn injury), wounds were analyzed for IFN-γ by mincing a 10-mm punch biopsy of the CK allograft in phosphate buffered saline. Biopsies were then sonicated, vortexed, and centrifuged at 1500 rpm for 5 minutes. Supernatants were collected and frozen at −80 C. until the IFN-γ assay was performed. Unburned skin from the right ear was similarly processed. Serum IFN-γ was determined by collecting blood through retro-orbital puncture, allowing the clot to separate, centrifuging the serum, and freezing the supernatant at −80 C.

Determination of Interferon-γ Levels

Interferon-γ levels in CK allografts, unburned skin, and serum were measured with a commercially available ELISA kit (Endogen Inc., Boston, MA), which is sensitive and specific for murine IFN-γ between concentrations of 15 and 3000 pg/mL. Samples were tested in duplicate and compared with known IFN-γ standards.

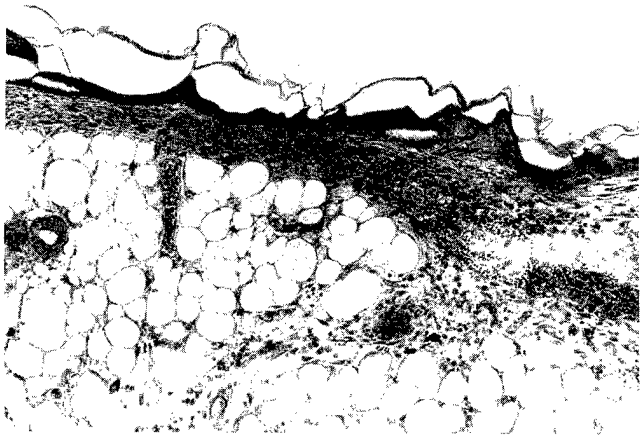

Figure 1. Photomicrograph (×100 magnification) of a CK allograft 5 days after burn injury and 3 days after wound excision and grafting. This CK allograft is closely adherent to musculoskeletal fascia (under which unstained muscle fibrils are visible) and contains a stratified keratinocyte layer with a basal component. Also apparent is a newly formed keratin sheet not observed in ungrafted keratinocyte cultures.

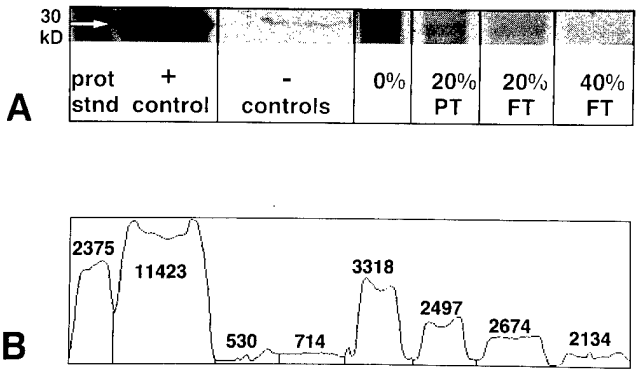

Figure 2. (A) Western immunoblot depicting MHC class II alloantigen expression after burn injury and grafting. prot stnd: low molecular weight protein standards; + control: C57BL/6 splenocytes; −controls: CBA splenocytes and CBA skin; 0%: sham burn; 20% PT: 20% total body surface area partial-thickness burn; 20% FT: 20% total body surface area full-thickness burn; 40% FT: 40% total body surface area full-thickness burn. (B) Video densitometrogram relating absolute pixel density of individual protein bands. Burn injury decreased alloantigen expression as burn size increased.

Statistical Analysis

Mean MHC class II antigen expression was compared between burn groups by analysis of variance and Student's t test. Mean IFN-γ levels in grafted wounds, serum, and unburned skin were compared between burn groups by Fisher's protected least significant difference (LSD). Each burn group contains a sample size of approximately seven surviving animals.

RESULTS

Animal Survival

The graduated severity of our burn model can be appreciated by observing the mortality of each group. In these and other experiments, survival rates for each of the burn groups were as follows: 0% burn, 100% survival; 20% PT burn, 95% survival; 20% FT burn, 90% survival; 40% FT burn, 65% survival. In this study, autopsies of the animals who did not survive after postburn day 5 showed mesenteric edema and ischemia; no wound infections were grossly apparent.

Histochemical Studies

All of the wounds had either macroscopic or microscopic evidence of CK allograft persistence 3 days after

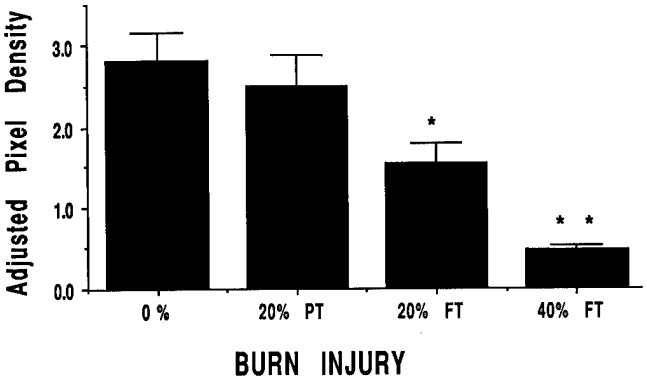

Figure 3. The effect of burn size on MHC class II antigen expression in CK allografts 3 days after excision and grafting. Each group contains approximately seven surviving animals. Immunoblots were scanned with video densitometry to quantify alloantigen expression, which is depicted graphically as mean adjusted pixel density. Error bars represent standard error of the mean. Both 20% FT and 40% FT burn injuries significantly inhibited class II alloantigen expression. *$p < 0.05$ *vs.* 0%; **$p < 0.001$ *vs.* 0%.

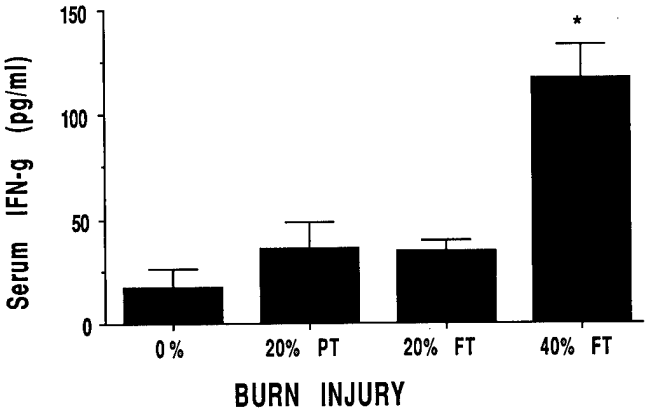

Figure 5. The effect of burn size on serum levels of IFN-γ. Although 20% PT and FT burns modestly increased serum IFN-γ, only 40% burn injury significantly increased serum levels of this cytokine. *$p < 0.01$ *vs.* 0%, 20% PT, 20% FT.

grafting. Acid-fuschin histology revealed the presence of acellular keratin sheets, stratification of keratinocytes with a basal layer, and good apposition of the CK allografts to the chest wall, as illustrated in Figure 1. Burned animals tended to have a more pronounced mononuclear cell infiltrate between the CK allografts and muscle fascia, consistent with the increased inflammation expected of burn injury.

MHC Class II Alloantigen Expression

To assess the induction and expression of MHC class II antigen *in vivo*, we performed Western immunoblots on allograft biopsies from mice whose burn wounds had been partially excised and covered with allogeneic cultured keratinocytes. We observed that burn injury decreased MHC class II antigen expression as a function of burn size. The immunoblot in Figure 2 demonstrates donor-specific class II protein expression from all four burn groups. Direct comparison with the accompanying video densitometrogram enables the viewer to quantify the intensity of each band, as measured by pixel density. In this experiment, positive controls included spleens and FT skin from C57BL/6 mice, whereas negative controls included spleens and FT skin from CBA mice, *in vivo* CK autografts, and *in vitro* C57BL/6 keratinocyte sheets.

As depicted in Figure 3, MHC class II alloantigen expression decreased as a function of burn depth and burn size. Mean antigen expression for each burn condition was determined by scanning protein bands with video densitometry, measuring absolute pixel density for each

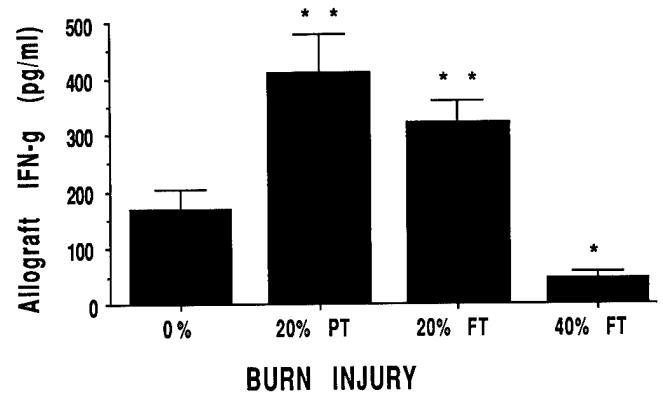

Figure 4. The effect of burn size on IFN-γ levels in grafted wounds. Both 20% PT and 20% FT injuries significantly increased wound IFN-γ, whereas 40% FT burns decreased wound IFN-γ. *$p < 0.05$ *vs.* 0%, 20% PT, 20% FT; **$p < 0.01$ *vs.* 0%, 40% FT.

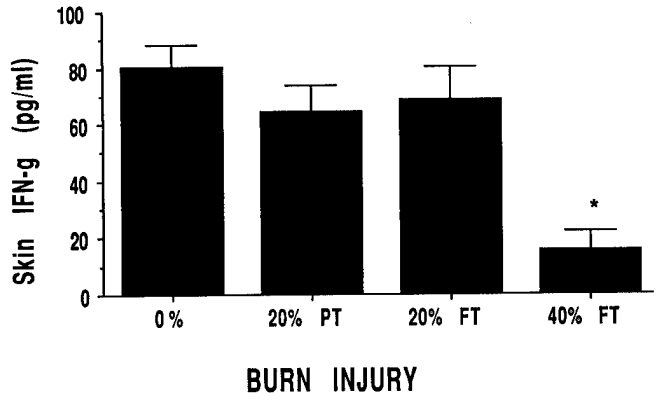

Figure 6. The effect of burn size on IFN-γ levels in unburned skin. Both 20% PT and 20% FT burns slightly decreased local levels of IFN-γ, but only 40% burn injury significantly inhibited levels of this cytokine in unburned skin. *$p < 0.01$ *vs.* 0%, 20% PT, 20% FT.

band, adjusting for gel-to-gel variability by dividing pixel density by background density, and averaging these adjusted densities for each group. Mean antigen expression for each burn condition was as follows: 0% sham, 2.81; 20% PT burn, 2.49; 20% FT burn, 1.54; 40% FT burn, 0.47. Both 20% FT and 40% FT burn injuries significantly decreased expression of MHC class II alloantigen compared with the sham controls ($p < 0.001$). A 20% PT burn also inhibited class II expression, but this difference was not statistically significant. This relationship between burn injury and alloantigen expression corresponds with the immunoblot from Figure 2, in which biopsies from all four groups were developed on the same gel.

Tissue Levels of IFN-γ

With evidence that burn injury decreases antigen expression in CK allografts, we decided to study the mechanism responsible for the induction and regulation of MHC class II antigen. Interferon-γ, which has been implicated as a critical mediator of class II antigen expression, was measured after thermal injury in allografted wounds, serum, and unburned skin. Both 20% PT and 20% FT burns significantly increased wound levels of IFN-γ, whereas a 40% injury significantly decreased levels of this cytokine compared with the unburned, grafted controls (Fig. 4). Mean wound levels of IFN-γ (pg/mL) were as follows: 0% sham, 167.4; 20% PT burn, 409.3; 20% FT burn, 322.6; 40% FT burn, 47.7. Although a minor burn injury increased levels of wound IFN-γ, burn injury inhibited wound IFN-γ as the severity of injury increased, corresponding to impaired alloantigen expression.

In this model of burn injury, serum levels of IFN-γ increased as the size of the burn injury increased. Mean serum IFN-γ levels for 0%, 20% PT, 20% FT, and 40% FT burns were 17.9, 36.1, 34.8, and 117.0, respectively. Although both 20% PT and 20% FT burns modestly increased serum IFN-γ, 40% FT burn injury increased levels of this cytokine sixfold (Fig. 5). Despite the finding that serum levels of IFN-γ increased as a function of burn size, we observed that IFN-γ levels in unburned skin decreased as burn size increased. Mean skin levels of IFN-γ for 0%, 20% PT, 20% FT, and 40% FT burns were 80.4, 64.1, 68.5, and 15.2, respectively. Both 20% PT and 20% FT burn injuries slightly decreased levels of skin IFN-γ, and a 40% FT injury significantly decreased levels of this cytokine (Fig. 6).

In summary, major thermal injury increases systemic circulating levels of IFN-γ but decreases delivery and/or local production of this cytokine. Although wound IFN-γ increases after minor burn injury, the profound decrease in wound and skin IFN-γ after major burn injury corresponds with diminished class II alloantigen expression. The relationship between burn injury, alloantigen expression, and IFN-γ levels are shown in Table 1, which provides a summary of these data.

DISCUSSION

In this study, we provided evidence that burn injury decreases the immunogenicity of CK allografts. Although keratinocytes do not normally express MHC class II antigen, exposure to IFN-γ induces *de novo* expression of class II antigen and imparts on keratinocytes the ability to initiate an alloimmune response.[15] Burn injury, however, appears to inhibit induction of MHC class II antigen in a burn-size-dependent manner. Furthermore, decreased expression of MHC class II antigen in large burns corresponds with diminished wound and skin levels of IFN-γ. The implication of these findings is that the immunosuppression of burn injury decreases the antigenicity of CK allografts, which might extend the long-term survival of these grafts and improve their potential as a permanent skin replacement.

The early excision and closure of massive burn wounds increases patient survival and improves functional outcome[16] but remains a significant challenge for burn care providers. In 1983, Hefton et al. reported the use of CK allografts in three patients who underwent tangential excision of deep PT injuries.[1] Early enthusiasm for these grafts was based on the principles that CK allografts could be grown and stored before injury,[17] that the grafts function as a bioactive occlusive dressing,[18] and that CK allografts have limited immunogenicity.[19]

Cultured keratinocyte allografts have also been promoted in the treatment of nonburn conditions, such as epidermolysis bullosa[20] and venous stasis ulcers,[21] with the rationale that these grafts secrete numerous growth factors that guide keratinocyte migration, increase angiogenesis, and participate in the formation of the extracellular matrix.[22] Two recently published, controlled, blinded trials suggested that CK allografts may be useful in management of skin graft donor sites by reducing pain and accelerating re-epithelialization. Although allogeneic keratinocytes did not survive permanently, CK allografts decreased wound healing from 14 to 8 days,[23] reduced the interval required between repeated donor site harvests, and improved the quality of the reharvested skin.[24]

However, numerous economical and technical limitations have prevented the widespread acceptance of CK allografts as a biologic material to be used in burn wound coverage. Cultured epidermal sheets cost approximately $13,000 per 1% body surface area covered and require multiple applications because of unpredictable graft take.[25] Cultured keratinocyte allografts may develop a

Table 1. THE EFFECT OF BURN SIZE ON ALLOANTIGEN EXPRESSION AND IFN-γ LEVELS IN ALLOGRAFTED WOUNDS, SERUM, AND UNBURNED SKIN

Burn Size (% TBSA)	MHC Class II Alloantigen Expression (relative pixel density)	IFN-γ Levels (pg/mL)		
		Wound	Serum	Skin
0	2.81 ± 0.34	167.4 ± 36.0	17.9 ± 8.8	80.4 ± 8.0
20 PT	2.49 ± 0.38	409.3 ± 69.5	36.1 ± 12.8	64.1 ± 9.5
20 FT	1.54 ± 0.25	322.6 ± 35.4	34.8 ± 5.5	68.5 ± 11.7
40 FT	0.47 ± 0.08	47.7 ± 10.9	117.0 ± 16.0	15.2 ± 6.9

TBSA = total body surface area; PT = partial thickness, FT = full thickness.
Values are mean ± SEM.

"neodermis" after several months,[26] but the lack of a dermal element increases the mechanical fragility of recently grafted keratinocytes. Furthermore, allogeneic keratinocytes, when placed on PT wounds, are gradually replaced by host keratinocytes, as determined by DNA fingerprinting,[27] and may be effective only when the wound can be repopulated by keratinocytes from deeper dermal elements. The fate of CK allografts when used to cover fascia or granulation tissue remains unknown.

Although CK allografts do not contain passenger leukocytes, these grafts are more immunogenic than originally believed. Keratinocytes express MHC class II antigen in various autoimmune diseases,[28] after exposure to IFN-γ *in vitro,*[9] and when grafted as epidermal sheets.[8] Using a model of second-set rejection originally described by Medawar in 1944,[29] we have demonstrated that CK allografts and FT allografts sensitize the unburned host with equal efficacy.[8] Prior exposure to either graft decreases second-set allograft survival from 13 to 9 days. This priming occurs through activation of cytotoxic T lymphocytes and has possible clinical implications in patients who need extensive, repeated coverage with CK allografts.

Because burn injury produces numerous, specific defects in cell-mediated immunity, such as suppression of T-lymphocyte activation,[30,31] we suspected that burn injury might undermine the process of sensitization and ameliorate the immunogenicity of allogeneic keratinocytes. We recently reported that burn injury selectively interferes with priming by CK allografts compared with FT allografts.[12,13] Second-set rejection in burned mice who had received CK allografts was significantly delayed. The current study examined the effect of burn injury on the antigenicity of CK allografts, and we propose that impaired priming by allogeneic keratinocytes may be due to down-regulation of MHC class II antigen, which is critical in initiating the rejection response.

A possible mechanism for diminished alloantigen expression may be related to altered cytokine levels after burn injury. Interferon-γ, which is a 20- to 25-kD polypeptide produced by activated T lymphocytes, normally up-regulates MHC expression, making allografts, virally infected cells, and tumors more susceptible to cytotoxic T-lymphocyte–mediated lysis.[32] Keratinocytes are not privileged from this process and can serve as both stimulators and targets of the immune response.[33] Although they lose T-cell activating ability in culture,[34] keratinocytes develop MHC class II antigens and express increased class I antigens when stimulated with IFN-γ.[15] However, interleukin-1, tumor necrosis factor-α, and prostaglandin E_2, which are elevated after thermal injury,[35,36] have been shown to either down-regulate the expression of class II antigen or antagonize the effects of IFN-γ.[37–39]

The effect of burn injury on IFN-γ production, though, has not been fully defined. Our observation that burn injury decreases tissue levels of IFN-γ is consistent with previous research that implicates decreased IFN-γ as a cause of postburn immunosuppression. In a series of classic experiments, Suzuki and Pollard demonstrated a biphasic depression in T-cell production of IFN-γ 3 to 5 days and 3 to 5 weeks after burn injury.[40,41] Furthermore, the size of the burn wound was inversely correlated with the ability of T cells to generate IFN-γ *in vitro.*

Evidence for the importance of this cytokine in restoring immunocompetence after thermal injury and trauma can be noted in the ability of IFN-γ to decrease bacterial translocation in mice,[42] improve natural killer cell function in rats,[43] and decrease infection-related deaths in humans.[44] Several investigators have suggested that susceptibility to infection after trauma may be related to impaired antigen detection through down-regulation of host MHC class II antigen.[45,46] Using flow cytometry to quantify human leukocyte antigen-DR expression, these authors have shown that depressed *in vitro* production of IFN-γ correlates with reduced monocyte class II expression, which was noted as early as 24 hours after injury and lasted for 2 weeks. This report is

consistent with our observation that a 40% burn injury inhibits class II alloantigen expression as well as allograft levels of IFN-γ.

Elevated graft levels of IFN-γ after a minor PT burn injury may be due to local proinflammatory effects of the burn wound. Our finding that even minor burn injury inhibits class II expression, despite increased levels of IFN-γ, suggests that other important cytokines are involved in the regulation of class II alloantigen. Although speculative, one explanation is that tumor necrosis factor-α and prostaglandin E_2, both of which are increased after burn injury, may antagonize the effects of IFN-γ and down-regulate class II antigen expression.

Many studies have examined the effect of burn injury on serum cytokine levels, but very few have focused on systemic circulating levels of IFN-γ. One group has recently reported that in burn patients, infection rates and injury severity positively correlated with plasma levels of both IFN-γ and interleukin-6.[47] These data, combined with our observation that burn injury increases serum levels of IFN-γ but decreases skin levels, imply that this immunoregulatory cytokine is not available for local use, despite elevated plasma levels. Major burn injury, through undescribed mechanisms, may interfere with the local production of IFN-γ or may interfere with tissue delivery. The increased serum IFN-γ levels observed after burn injury may also reflect compensatory production of this cytokine by circulating lymphocytes in an effort to restore immunocompetence.

In conclusion, we report that a significant burn injury, with its associated immunosuppression, decreases the immunogenicity of CK allografts by inhibiting expression of MHC class II alloantigen. Furthermore, decreased expression of alloantigen corresponds with decreased levels of IFN-γ in grafted wounds. The decreased immunogenicity of CK allografts may minimize sensitization and allow for repeated applications in burn patients without alloantigen priming. Additionally, this decreased immunogenicity may improve the long-term survival of allogeneic keratinocytes and enable early, complete excision of FT burn wounds. Understanding and further manipulating the mechanism of alloantigen induction may someday permit the use of CK allografts as a definitive, permanent, biologic skin replacement.

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Discussion

DR. BASIL A. PRUITT, JR. (Fort Sam Houston, Texas): Dr. Hultman and his colleagues from our President's Department of Surgery have identified an association between suppression of donor specific major histocompatibility complex (MHC) class 2 alloantigen expression in cultured keratinocyte allografts and severity of burn injury. They have postulated that the MHC class 2 antigen suppression is due to a decrease in wound and skin levels of interferon-gamma and is associated with delayed rejection of cultured keratinocytes. Additional information is needed to evaluate the findings and assess the authors' conclusions.

The measurements of MHC class 2 antigens were made 3 days after the cultured allografts were applied. I wonder whether that suppression decreases with time following excision and application as the local blood supply increases.

If, as you propose, decreased wound interferon-gamma is related to decreased MHC class 2 alloantigen expression, how can the association of increased interferon-gamma levels with decreased graft MHC class 2 alloantigen expression in the animals with a 20% full- thickness burn be explained?

In that same vein, is it possible that the suppression of MHC class 2 alloantigens in grafts applied to the full-thickness burns simply reflects heat-induced thrombosis and the avascularity of such burns as compared with partial thickness injuries?

Have you had the chance to measure interferon-gamma production by lymphocytes, which, as you note in your manuscript, may be the source of the increased serum levels you observed?

You have recently reported that even partial excision of a burn wound partially corrects burn-induced immunosuppression. In light of that, is the postburn decrease in cultured keratinocyte allograft expression of class 2 antigens reduced by partial excision or completely eliminated by total excision?

Lastly, you mention interleukin-1, tumor necrosis factor-alpha, and prostaglandin E_2 as possible mediators of the observed effects of burn injury. Do you have any measurements of those cytokines to support that contention? And do those measurements identify a cytokine or cytokines which bear a constant relationship to reduced class 2 alloantigen expression and might therefore be used therapeutically to delay rejection indefinitely?

DR. DAVID N. HERNDON (Galveston, Texas): I would like to congratulate the authors on an excellent presentation. I would

like to amplify some of Dr. Pruitt's comments and ask two questions of my own.

Skin graft biopsies were taken only 3 days after application and 5 days postburn. Alloantigen expression was markedly decreased at that time. What would a regular skin graft other than a tissue culture skin graft do at 3 to 5 days after application?

Rejection is not seen in skin grafts at 3 to 5 days postapplication. Rejection does not occur until, as Medawar showed, 1 to 2 weeks after application of a regular skin graft in immunocompetent host. In burn injuries over 40% total body surface area, skin grafts from cadavers and totally dissimilar people are not rejected until 3 to 5 weeks postapplication. The phenomena that you are examining occurs at 3 to 5 days after injury. The critical question is, what happens over time? You have alluded to some classic studies that have been done earlier that show interferon-gamma production in thermal injury is decreased in a biphasic way 3 to 5 days postinjury, and 3 to 5 weeks after major burn injury. Perhaps these are related phenomena: the ones that you are studying at 3 to 5 days after injury and those that are more clinically applicable.

The last question I would like to ask is, do these findings have potential for clinical application? The titillating suggestion that we might use tissue culture allogeneic cells to cover extensive burns may be a fairly large leap. Tissue culture grown cells have many problems other than rejection. But cadaver-grafted full-skin sheets would be of greater utility in coverage of extensive burns. I am wondering if you might comment how these results might impact on that clinical possibility.

DR. ANTHONY A. MEYER (Closing Discussion): I would like to thank Dr. Pruitt and Dr. Herndon for their comments.

With respect to Dr. Pruitt's questions and comments, we chose 3 days after grafting with cultured keratinocyte allografts because in some of our previously reported studies, 3 days after grafting seems to be the time for maximum class 2 expression. In fact, if you go a period of time after 3 days, class 2 expression seems to fall off a bit. We have not seen it go to zero.

Dr. Pruitt brought up the important question of why, when you have a 20% full-thickness burn and an increase in local allograft production of interferon-gamma, do you then see a decrease in class 2 expression?

As pointed out, there are other cytokines that are important in class 2 antigen expression that have been studied and noted by other individuals. Tumor necrosis factor-alpha, interleukin 1, prostaglandin E_2, all contribute to this. Interferon-gamma is the one we chose because it is more classically used as a moderator of keratinocyte class 2 antigen expression.

We do have some preliminary data that show that tumor necrosis factor-alpha, which down-regulates class 2 antigen expression, is increased after burn injury, and that may represent why, in the 20% full-thickness group, you do get an unexplained decrease in class 2 expression despite an increase in local production of interferon-gamma.

Dr. Pruitt then asked if we had measured interferon-gamma production by lymphocytes. No, we have not. But it would be a good thing to pursue, I believe. He asked if after burn reduction by partial or even total excision, will change our class 2 antigen expression? That is not something we have done. This is only partial excision, and it would be worthwhile trying to do this on completely excised animals. We know that complete excision does have different effects on immune function, but we have not looked at it yet at this point.

Dr. Herndon brought up two important points. He stated that the full-thickness skin is a concern and asked: Why do we get this with keratinocyte allografts and not full-thickness allografts and pointed out that full-thickness cadaveric grafts have been used for a long period of time.

The reason we chose cultured keratinocyte allografts is it is a single-cell population that can be carefully studied and manipulated. With full-thickness skin, you have the components of not only keratinocytes but fixed tissue macrophages, Langerhans' cells, endothelial cells, and many other types of cells. Therefore, it is difficult to say one cell type would be solely responsible for sensitization.

Interestingly, despite the fact that it sometimes takes more than a month to reject full-thickness skin, it only takes is 2 to 4 hours of contact to sensitize an animal. You can then remove the full-thickness allograft and the animal is sensitized to subsequent second set rejection. So it does not require a long period of exposure. All it requires is 2 to 4 hours of direct contact on the wound.

Dr. Herndon brings up the question of clinical relevance. I think this is important. The problems with cultured keratinocytes are significant. We are continuing to work on possible ways to solve some of these problems. I would point out that histocompatibility antigens, which are part of what we are studying and part of the rationale why we are studying it, are not only important in possible use of cultured keratinocytes or full thickness allografting for burns, but they are also involved in processing viral and neoplastic antigen targets as well.

It is important that we understand the mechanism of antigen expression if we are able to try to manipulate this as a way to either get full-thickness or cultured skin allografts to be permanent coverage. Potentially we could develop a "knock-out" or a class 2-deficient skin cell line or potentially knock out other important alloantigens that would permit universal donor type keratinocytes that could be grown in large production and used to graft individuals.

I think that there is still some potential but there is a considerable amount of work to be done for this topic.

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Vol. XLVII, 1996

ALLOGENEIC KERATINOCYTES DEFICIENT IN CLASS II ANTIGENS FAIL TO PRIME THE HOST FOR ACCELERATED SECOND-SET REJECTION OR ENHANCED T LYMPHOCYTE CYTOTOXICITY

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FULL-THICKNESS (FT) AND CULTURED KERATINOCYTE (CK) allografts have been used as temporary skin replacements in patients with massive burns, but these grafts are ultimately rejected after restoration of host immunocompetence.[1] Genetic engineering has recently permitted the creation of knock-out mice deficient in class I ($I^{-/-}$) and class II ($II^{-/-}$) histocompatibility antigens.[2-4] This study examines the immunogenicity of such grafts to determine if keratinocytes from these donors could be utilized for permanent wound coverage.

MATERIALS AND METHODS

In the first experiment, host sensitization to donor foreign antigens was assessed by second-set rejection. CBA mice (n = 111) were

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Median survival time of second-set tail allografts (days)

Flank graft	B6 allograft	I $^{-/-}$ B6 allograft	II $^{-/-}$ B6 allograft	CBA autograft
CK	9	11*	12*	13*
FT	9	9	11*	

*$P < 0.05$ vs. B6 FT.

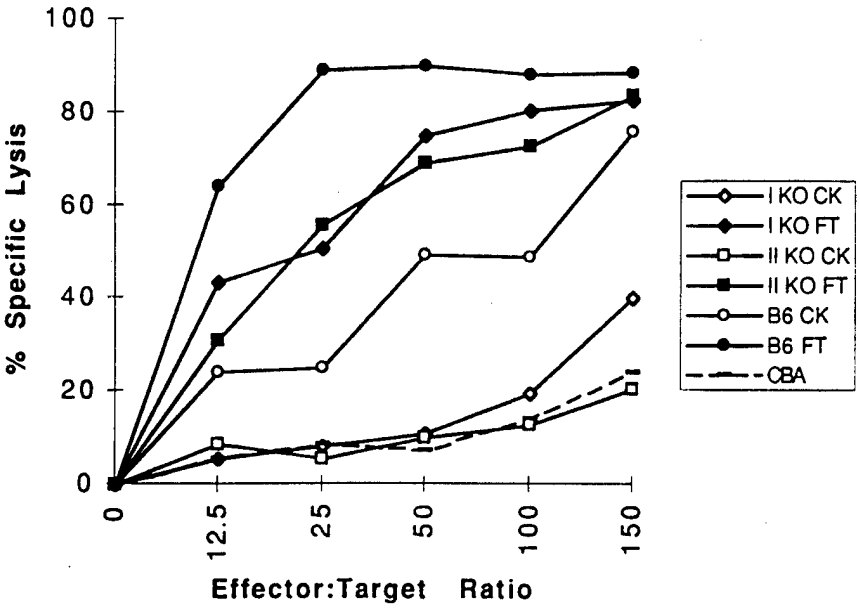
primed with flank grafts consisting of C57BL/6 (B6) FT and CK allografts (+ controls), I $^{-/-}$ FT and CK B6 allografts, II $^{-/-}$ FT and CK B6 allografts, and CK CBA autografts (− control). Three weeks later, hosts were challenged with normal tail allografts that were observed for second-set rejection. Median survival time of tail allografts was compared between groups by Wilcoxon rank and χ^2 analysis. In the second experiment, cytotoxic T lymphocytes (CTLs) were harvested from CBA mice ($n = 28$) 3 weeks after flank grafting. CTL effectors were tested on radiolabeled, allogeneic targets (EL-4 cells) at varied ratios in a ^{51}Cr release assay. Percent specific lysis of targets was determined via a γ-counter and was used as an index of CTL function. Dilution curves of CTL activity were compared by ANOVA.

RESULTS

Hosts primed with CK or FT B6 allografts demonstrated accelerated rejection of second-set tail grafts (9 days), compared to hosts covered with CBA autografts (13 days) (see Table). FT knock-out skin was more immunogenic than CK knock-out grafts. II $^{-/-}$ allografts were considerably less immunogenic than I $^{-/-}$ allografts, resulting in delayed rejection of second-set grafts. Mice grafted with II $^{-/-}$ keratinocytes showed no evidence of sensitization by second-set rejection (12 vs. 13 days, NS). Hosts primed with normal allografts or FT knock-out allografts generated considerable CTL activity against allogeneic targets (see Fig). In contrast, mice grafted with normal, allogeneic keratinocytes displayed moderate CTL alloreactivity, whereas mice grafted with autografts or knock-out keratinocytes demonstrated negligible CTL function.

DISCUSSION

Although full-thickness knock-out skin appears to retain substantial immunogenicity, cultured keratinocytes deficient in class II histocompatibility antigens fail to prime the host for accelerated second-set rejection and do not elicit a host CTL response. This lack of immunogenicity may result in delayed rejection of allogeneic knock-out keratinocytes. Further manipulation of these histocompatibility anti-



CTL alloreactivity of grafting groups at varied E:T ratios. CBA mice (n = 28) were flank grafted with class I knock-out cultured keratinocytes (I KO CK), class I knock-out full-thickness skin (I KO FT), class II knock-out keratinocytes (II KO CK), class II knock-out skin (II KO FT), normal allogeneic keratinocytes (B6 CK), normal allogeneic skin (B6 FT), and normal syngeneic keratinocytes (CBA). Three weeks after grafting, splenocytes were harvested from these groups and used as CTL effectors in a standard ^{51}Cr release assay. Radiolabeled, allogeneic EL-4 targets were mixed with effectors at varied ratios. Percent specific lysis of targets was determined via a γ-counter and was used as an index of CTL function. Mice primed by B6 FT, I KO FT, and II KO FT grafts generated the greatest CTL alloreactivity, followed by hosts primed with B6 CK grafts. Animals covered with CBA, I KO CK, and II KO CK grafts displayed negligible CTL alloreactivity.

gens, via disruption of both class I and class II molecules, may permit the indefinite survival of these grafts in patients requiring massive wound excision and coverage.

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0079-6061/96/4101-0051$03.00/0
The Journal of Trauma: Injury, Infection, and Critical Care

Vol. 41, No. 1
Printed in the U.S.A.

Allogeneic Fibroblasts Used to Grow Cultured Epidermal Autografts Persist in Vivo and Sensitize the Graft Recipient for Accelerated Second-Set Rejection

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Introduction: **Cultured epidermal autografts (CEAs) have been used for wound coverage in patients with massive burns and other skin defects. However, CEAs often display late breakdown, which may be immunologically mediated and initiated by persistent foreign fibroblasts used as a feeder layer to optimize keratinocyte growth. This study investigates whether these fibroblasts, previously shown to persist in vitro, survive after grafting and induce host sensitization to alloantigen.**

Methods: **CEAs from CBA donors (H-2^k) were grown on allogeneic NIH 3T3 (H-2^q) or syngeneic LTK (H-2^k) fibroblasts, which were removed by trypsinization 7 days later. CBA mice (n = 85) were flank-grafted with NIH allografts (positive control), CEA/3T3s, CEA/LTKs, or CBA autografts (negative control). Hosts were challenged with second set NIH tail allografts 3 weeks later. Median graft survival was compared between groups by Wilcoxon rank and χ^2 analysis. Additional CBA mice (n = 15) received CEAs that were biopsied 0, 4, and 8 days after grafting. The presence of allogeneic fibroblasts was determined by Western immunoblotting, using KL295, a monoclonal antibody that recognizes H-2^q (but not H-2^k) class II histocompatibility antigens.**

Results: **Allogeneic fibroblasts persisted after grafting but decreased over time, as determined by alloantigen expression on Western immunoblots. Accelerated tail graft rejection occurred in hosts primed by NIH allografts (9 days, $p < 0.05$), as well as by CEAs grown with an allogeneic (10 days, $p < 0.05$) but not a syngeneic feeder layer (12 days, NS). Mice receiving flank autografts rejected second set tail allografts at 12 days.**

Conclusions: **Immunogenic fibroblasts used to grow CEAs survive in vivo and sensitize the graft recipient for accelerated second-set rejection. These persistent cells may initiate an inflammatory response that may result in late graft breakdown and limit the utility of CEAs grown with a foreign fibroblast feeder layer.**

Key Words: **Burn injury, Skin replacement, Cultured epidermal autograft.**

Cultured epidermal autografts (CEAs) have been proposed as a biologic, functional skin replacement in patients with massive thermal injury.[1–3] Wound coverage in these patients represents a significant technical problem, because of limitations in the quality and quantity of autogenous, partial-thickness skin that can be harvested. CEAs are particularly attractive because these grafts permit the 10,000-fold expansion of donor keratinocytes, but the 3-week cultivation period required for their growth, combined with their unpredictable take, their lack of a dermal component, and their excessive cost, have dampened enthusiasm for this biotechnology.[4,5] Furthermore, many investigators have described the phenomenon of late graft loss, which appears to be independent of mechanical and infectious causes and may have an immunologic component.[6,7] Clinically, previously healed wounds undergo blistering and ulceration, sometimes resulting in total autograft destruction.

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Supported in part by U.S. Army Grant DAMD 17–91-Z-1007 and the North Carolina Jaycee Burn Center. The viewpoints expressed in this paper represent those of the authors and not necessarily those of the U.S. Army or the Department of Defense.

Presented at the 55th Annual Meeting of the American Association for the Surgery of Trauma, September 27–30, 1995, Halifax, Nova Scotia, Canada.

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We have recently reported that growth-arrested, xenogeneic fibroblasts routinely used to accelerate keratinocyte growth in vitro survive after passage to secondary and tertiary cultures, as evidenced by flow cytometry and Western immunoblotting.[8] Such persistence occurs despite established techniques of feeder layer removal. The purpose of this study is twofold: (1) to determine if foreign fibroblasts used to cultivate CEAs persist in vivo after grafting, and (2) to determine if these fibroblasts sensitize the host to foreign histocompatibility antigens. This is clinically important because immunogenic fibroblasts could initiate an inflammatory response that induces local CEA destruction and, possibly, complete graft breakdown.

MATERIALS AND METHODS

Experimental Design

To determine whether fibroblasts used to cultivate CEAs persist in vivo after grafting, we first grew CBA keratinocyte sheets with either a syngeneic (LTK fibroblasts) or an allogeneic (3T3 fibroblasts) feeder layer. CEAs were then flank-grafted onto CBA hosts (n = 15) and biopsied 0, 4, and 8 days after application. Wound samples were assessed for the presence of fibroblast-specific class II histocompatibility antigens by Western immunoblotting. In the second series of experiments, we used a model of second-set rejection to determine whether or not allogeneic fibroblasts survive to prime CEA recipients for accelerated rejection of second-set

TABLE 1. Summary of keratinocyte and fibroblast haplotypes

	Murine Haplotype	
	H-2^k	H-2^q
Keratinocyte/graft donors	CBA	NIH Swiss
Fibroblast cell line	LTK	NIH 3T3

All graft recipients were CBA mice (H-2^k).

allografts. CBA hosts (n = 85) were randomized to receive NIH full-thickness (FT) allografts (positive control), CBA FT autografts (negative control), intraperitoneal (IP) 3T3 fibroblasts (putative positive control), or CBA CEAs grown on a syngeneic feeder layer (putative negative control) or an allogeneic feeder layer (experimental group). Three weeks later, mice were challenged with FT second-set tail allografts, which were observed for rejection. Our hypothesis was that animals exposed to FT allografts, allogeneic IP fibroblasts, and CEAs cultivated with an allogeneic feeder layer would mount a more vigorous rejection response to second-set allografts than groups not exposed to alloantigen initially.

Animal Protocols

Eight- to 10-week-old, 20 gram, female CBA mice (H-2^k haplotype) (Harlan, Inc., Indianapolis, Ind) were used as autograft donors and graft recipients. Age-, weight-, and gender-matched NIH Swiss mice (H-2^q haplotype) (Harlan) were used as allograft donors. Table 1 summarizes the haplotypes of the keratinocyte/graft donors and fibroblast cell lines used in these experiments. All animal protocols had previously been approved by the University of North Carolina Committee on Animal Research and conformed to experimentation standards established by the National Institute of Health.

Keratinocyte Cultures

CEAs were cultivated based on methods originally described by Rheinwald and Green.[9] Except where noted, all tissue culture media were obtained from the Lineberger Cancer Center (Chapel Hill, NC). Tail skin was harvested from CBA donors, cleaned with 70% ethanol, and stored overnight at 4°C in Dulbecco's modified Eagle media (DMEM) and 0.1% penicillin/streptomycin. After washing the tails in Hank's buffered saline solution (HBSS), the skin was incubated for 2 hours at 37°C in 0.25% trypsin (Sigma Chemical Co., St. Louis, Mo). The epidermis was peeled apart from the dermis and vortexed to generate a single-cell keratinocyte suspension. Syngeneic (mouse connective tissue L cells (LTKs) ATCC CCL 1.3, H-2^k haplotype) and allogeneic (NIH 3T3 Swiss albino embryo fibroblasts (3T3s) ATCC CRL 1658, H-2^q haplotype) fibroblasts were used as feeder layers and were growth-arrested by exposing these cells to 4 μg/mL mitomycin-C for 45 minutes at 37°C. Fibroblasts were washed three times with HBSS, liberated from tissue culture plates with 0.1% trypsin, and suspended in plating media consisting of DMEM and Ham's F-12, 5% fetal bovine serum (Hyclone Laboratories, Logan, Utah), insulin 5.0 μg/mL, transferrin 5.0 μg/mL, and hydrocortisone 0.4 μg/mL (all from Sigma), cholera enterotoxin 0.01 μg/mL (Schwartz Mann/ICN Biochemical, Costa Mesa, Calif), and amphotericin 5.0 μg/mL (E. R. Squibb and Sons, Princeton, NJ).

Keratinocytes (4×10^6 cells total) were co-cultured at a 2:1 ratio with fibroblasts (2×10^6 cells total) in 15 mL of plating media for 3 days. Epidermal growth factor 10.0 ng/mL (Collaborative Research, Bedford, Mass) was added to the plating media for subsequent media changes, which were performed every 2 to 3 days. One week after initial plating, LTK and 3T3 fibroblasts were removed by differential trypsinization (0.1% trypsin with 0.02% ethylenediaminetetraacetic acid (EDTA) exposure for less than 5 minutes, followed by gentle rinsing with HBSS). Cultured keratinocytes were then allowed to reach confluence, which typically took 2 weeks. Three groups of CEAs were created using the technique described above: CEA − 3T3 (the feeder layer was selectively trypsinized and visibly removed), CEA + 3T3 (the feeder layer was not trypsinized and therefore not removed), and CEA + LTK (the feeder layer was left intact). The appearance of the keratinocyte sheets was assessed by filtered light microscopy and documented photographically.

Grafting Procedure and Antigen Priming

After reaching confluence, CEAs were released from culture dishes with the enzyme dispase (Boehringer Mannheim, Germany), put on petroleum-lined gauze with the basal surface exposed, and placed on the left flank fascia of anesthetized CBA hosts. The surrounding, circumferential wound edge was undermined to accommodate the gauze-protected grafts, which were covered with a hydrophilic dressing (Vigilon, C. R. Bond, Berkeley Heights, NJ) for 1 week and protected with a stapled fabric bandage. Full-thickness allografts and autografts were harvested from NIH and CBA mice, respectively, washed in HBSS, debrided of adipose, placed directly onto the left flank fascia of CBA hosts, and secured by skin staples and a similarly applied bandage. A sixth group of CBA mice was exposed to alloantigen by priming hosts with an IP injection of viable, nongrowth-arrested 3T3 fibroblasts (5×10^5 cells in 0.5 mL of plating media).

Western Immunoblotting

The persistence of allogeneic fibroblasts in vivo was determined by assessing CEAs for the presence of alloantigen, via Western immunoblotting, using a monoclonal antibody specific for class II alloantigen.[10] Four days before grafting, CEAs were treated with recombinant murine interferon-γ 10,000 U/mL (Genentech, San Francisco, Calif) to enhance class II antigen expression. Grafted wounds were biopsied 0, 4, and 8 days after grafting with a 3-mm punch probe. Protein lysates were prepared by solubilizing biopsies in 200 μL of 0.01 M Tris-HCL, pH 7.4, 0.15 M NaCl, 0.5% NP-40, and 1 mM phenylmethyl sulfonyl fluoride (PMSF). Samples were briefly vortexed, ice incubated for 15 minutes, centrifuged for 15 minutes at 4°C, stored at −80°C, and standardized for protein concentration immediately before immunoblotting. Twelve percent sodium dodecyl sulfate (SDS) polyacrylamide gels were cast with a Bio-Rad Model 360 vertical cell and a Model 361 casting chamber. Molecular-weight markers and detergent-extracted specimens were separated on one-

dimensional gels and transferred to nitrocellulose, which was blocked with 1% bovine serum albumin in Tris-HCL (pH 8.0). Immunoblots were then incubated with KL295, a murine monoclonal antibody that recognizes and binds to the denatured 30-kd β-chain of H-2^q and H-2^b (but not H-2^k) class II histocompatibility antigens. After primary antibody incubation, immunoblots were exposed to goat, anti-mouse, alkaline phosphatase-labeled, secondary antibody and developed to reveal protein bands.

Video Densitometry

Expression of fibroblast-specific, H-2^q alloantigen was quantified with video densitometry. Using Macintosh video recording equipment (Apple Computer, Cupertino, Calif), Western immunoblots were recorded as video images in Power Point (Microsoft, Redmond, Wash) and analyzed with Gel Capture software (NIH, Bethesda, Md). Class II alloantigen expression, as detected by the H-2^q/H-2^b specific KL295 monoclonal antibody, was objectively assessed by determining the pixel density for each protein band at 30 kd.

Second-Set Rejection

To test the immunogenicity of a persistent allogeneic feeder layer, the mice were challenged with second-set tail allografts, via a method previously reported.[11] Three weeks after IP priming with 3T3s (n = 16) or flank grafting with NIH FT (n = 16), CBA FT (n = 15), CEA + 3T3 (n = 10), CEA − 3T3 (n = 15), or CEA + LTK (n = 13) grafts, anesthetized mice received a 10-mm FT skin allograft (NIH) placed on the dorsal tail surface. This allograft was compared to a distally placed 10-mm FT skin autograft (CBA), which served as an internal control. Tail grafts were protected from organic debris and mechanical disruption by specially manufactured cylindrical glass tubes, which were removed after 3 days. Tail graft viability was assessed daily by two independent observers. Criteria for graft rejection were based on graft color, hair orientation, and scale integrity.

Statistical Analysis

Median survival time (MST) of second-set tail allografts was determined for each of the six groups and compared between groups by Wilcoxon rank and χ^2 analysis. Bonferroni's procedure was used to correct for multiple comparisons between groups. Statistical significance was assumed for differences of $p < 0.05$.

RESULTS

Human CEA Assessment

Our laboratory became interested in the hypothesis that immunogenic fibroblasts contribute to late CEA "rejection" based on our clinical observation that many patients at the North Carolina Jaycee Burn Center experienced late CEA breakdown, apparently independent of mechanical disruption or graft infection. To support and accelerate keratinocyte growth, human CEAs had been prepared with a murine 3T3 feeder layer, subsequently removed via differential trypsinization, using methods nearly identical to those of Rheinwald and Green.[9] Several weeks after successful CEA application and wound healing, many patients developed an inflammatory response that resulted in focal areas of graft blistering and ulceration, and occasionally complete CEA loss (Fig. 1). This phenomenon, combined with the significant cost of this technology, as well as the unpredictable initial graft take, prompted our laboratory to suspend the clinical use of CEAs until further investigation had elucidated the mechanism for breakdown and developed improved techniques of CEA cultivation.

Light Microscopy

In this experiment, murine CEAs were grown with either a syngeneic (LTK) of allogeneic (3T3) feeder layer. Figure 2 represents the appearance of a CEA sheet 7 days after initial plating; the keratinocyte monolayer consists of flat, cobblestone-type cells, while persistent fibroblasts are identified as raised, spindled-shaped cells found clustered as islands throughout the monolayer. Three days after differential trypsinization, or 10 days after plating, nearly all of the feeder layer fibroblasts have been removed, and keratinocyte sheets have reached confluence (Fig. 3). However, careful inspection of these sheets by light microscopy reveals a second population of cells, which appear to represent persistent allogeneic fibroblasts that could be transferred to the CEA recipient (Fig. 4).

Western Immunoblotting

To determine if these feeder layer fibroblasts persisted in vivo after grafting, we first cultivated CEAs with a feeder layer that was allogeneic to both the keratinocyte donor, as well as graft recipient. Western immunoblotting was performed to detect the presence of alloantigen in wound biopsies 0, 4, and 8 days after grafting. Alloantigen from 3T3 fibroblasts was detected using KL295, a murine monoclonal antibody that is specific for murine H-2^q (but not H-2^k) class II histocompatibility antigens.[10] As expected, CEAs grown with a 3T3 feeder layer that was not removed (CEA + 3T3) express a 30-kd band (2367 pixel units) in vitro that represents class II antigen from allogeneic fibroblasts (Fig. 5). Slightly diminished alloantigen expression is observed in CEAs where the 3T3 feeder layer has been visibly removed via trypsinization (1902 pixel units). Class II alloantigen expression decreases in vivo after grafting (4 days, 820 pixel units; and 8 days, 699 pixel units), but remains substantially greater than background nonspecific binding to syngeneic antigen of CBA splenocytes (266 pixel units). This suggests that viable, allogeneic fibroblasts survive after grafting, decrease over time, and are potentially immunogenic to the host as they express class II histocompatibility antigens. Positive controls, representing known class II alloantigen expression, include NIH splenocytes (10390 pixel units) and 3T3 fibroblast sheets incubated with interferon-γ (9304 pixel units). Multiple immunoblots from separate graft recipients were created and revealed similar results.

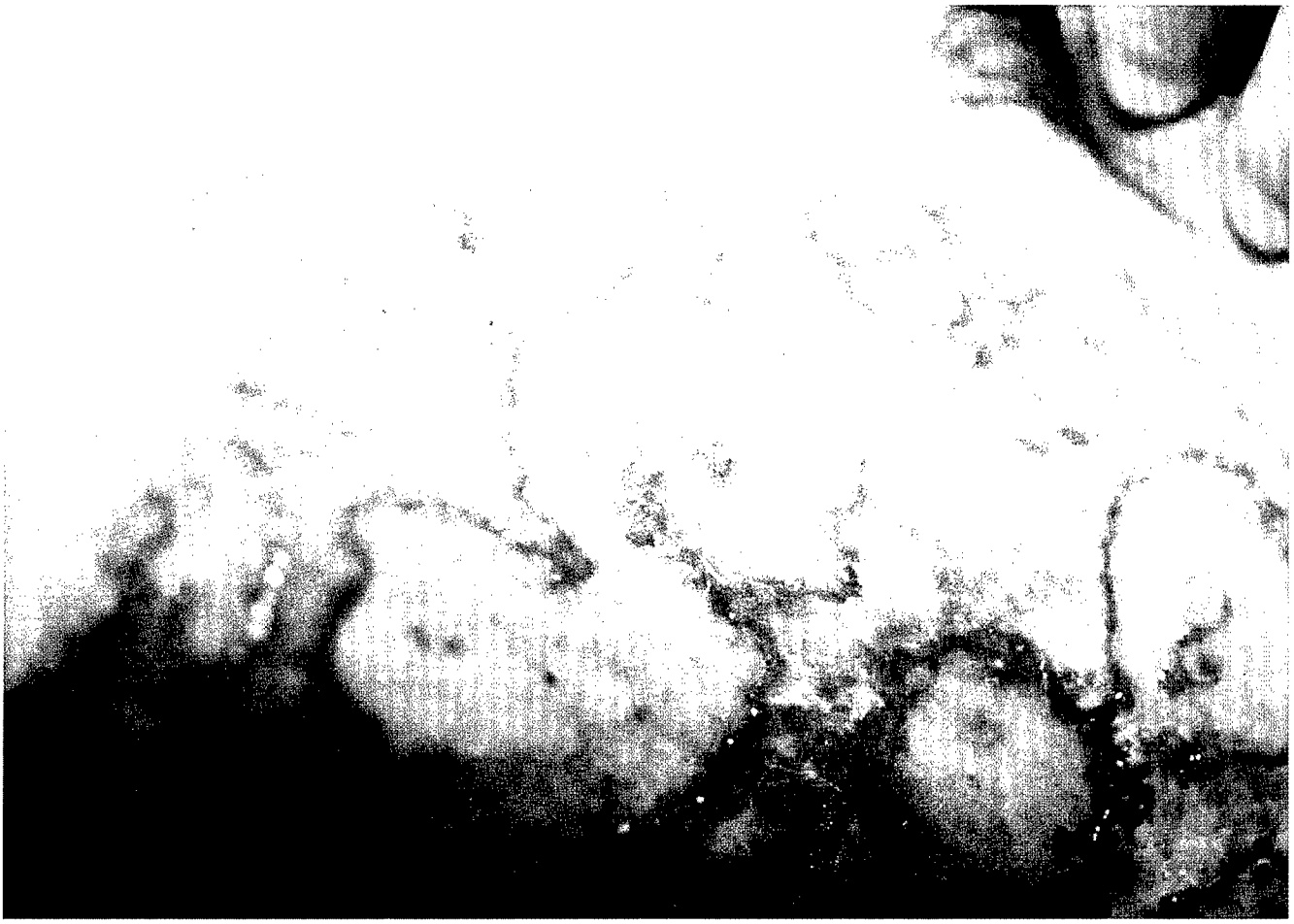

FIG 1. Appearance of late CEA breakdown, 20 days after grafting. The patient's left inguinal region is located in the upper right corner of the photograph. An intact portion of the CEA can be found along the patient's medial left thigh. Three weeks after successful engraftment, CEAs underwent spontaneous blistering and ulceration, with central areas of focal breakdown, as well as peripheral areas of advancing erythema. Within 48 hours, this portion of the CEA was completely destroyed, leaving only granulation tissue.

Second-Set Rejection

With evidence that feeder layer fibroblasts survive in vivo after grafting, we asked if such persistence resulted in host sensitization to alloantigen. To test the immunogenicity of CEAs grown with allogeneic fibroblasts, we used a model of second-set rejection, in which hosts previously exposed to alloantigen will generate a more vigorous rejection response when reexposed to that specific alloantigen.[12] In our particular experiment, we hypothesized that mice previously exposed to an allogeneic feeder layer would reject FT allografts

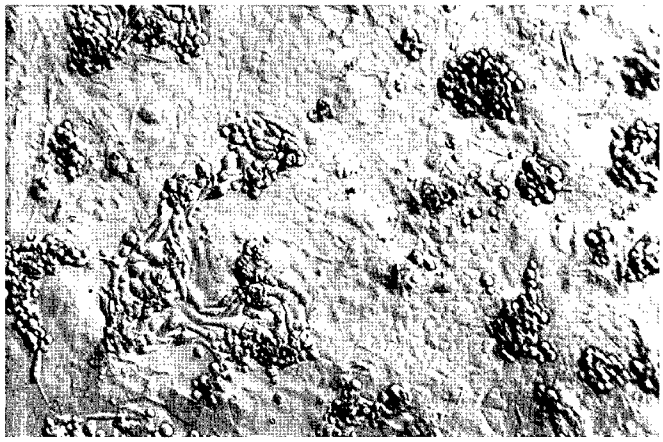

FIG 2. Photomicrograph of keratinocyte sheet, 7 days after plating. At least two populations of cells can be identified: the keratinocyte monolayer, which has a cobblestone-like, flat appearance, and residual feeder layer fibroblasts, which are spindle-like and raised relative to the keratinocytes. Magnification: 100×.

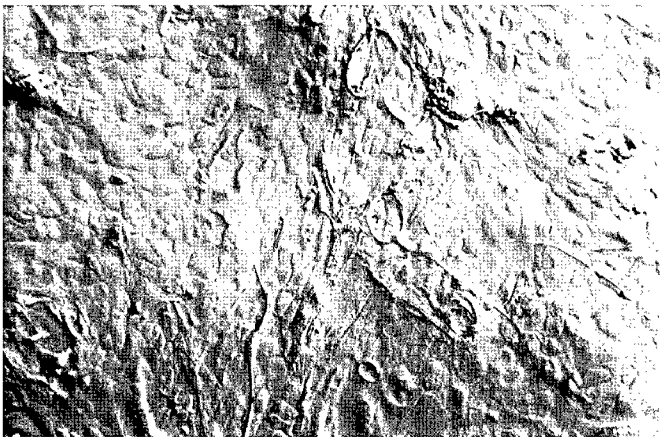

FIG 3. Photomicrograph of keratinocyte sheet, 10 days after plating and 3 days after differential trypsinization. The fibroblast feeder layer has been visibly removed, and the cultured keratinocytes have reached almost 100% confluence. Magnification: 100×.

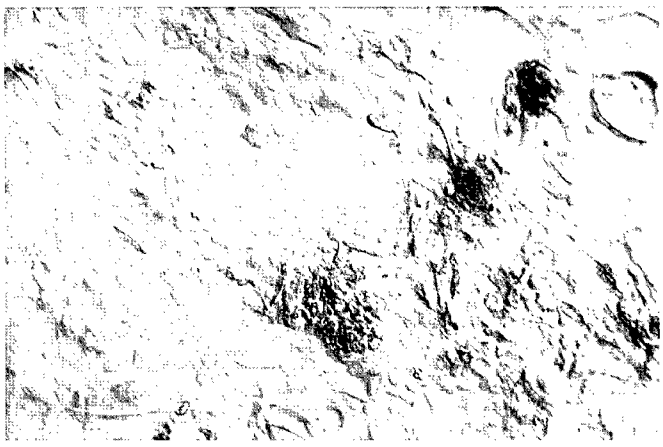

FIG 4. Photomicrograph of keratinocyte sheet, after differential trypsinization and before grafting. Although nearly all of the fibroblasts have been removed from this keratinocyte sheet, careful inspection by light microscopy reveals a second population of cells, which appear to be persistent fibroblasts surviving to engraftment. Magnification: 100×.

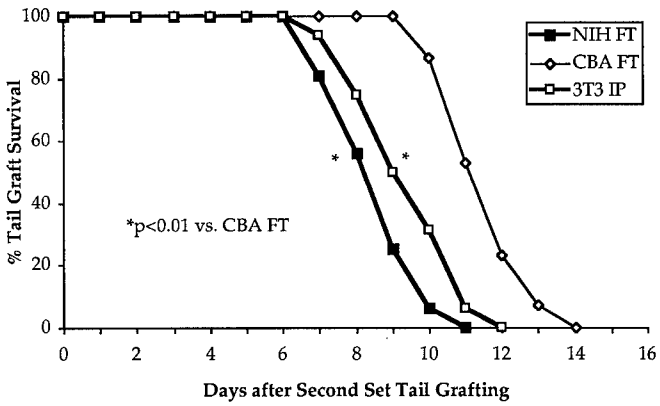

FIG 6. The effect of antigen priming on second-set rejection. H-2^k hosts primed with H-2^q alloantigen from both NIH full-thickness allografts and intraperitoneal 3T3 fibroblasts reject second-set tail allografts more vigorously than hosts grafted initially with syngeneic CBA skin ($p < 0.01$). Abbreviations: FT, full-thickness; IP, intraperitoneal.

more quickly than mice originally grafted with CEAs grown on syngeneic fibroblasts.

To investigate this query, we first had to characterize the effect of alloantigen priming, by observing the second-set rejection response of positive controls (NIH FT flank grafts), negative controls (CBA FT flank grafts), and putative positive controls (3T3 fibroblasts injected IP). Survival curves of second-set tail allograft survival are depicted in Figure 6. Mice primed with NIH FT flank grafts and IP 3T3 fibroblasts rejected tail allografts at 9 and 9.5 days, respectively, compared to mice flank grafted with autografts, who rejected second-set tail allografts after 12 days ($p < 0.01$). There was no statistical difference between the NIH FT and 3T3 FB groups. Median survival time (MST) of tail allografts, MST range, and sample size are listed in Table 2.

Three experimental CEA groups (CEA + 3T3, CEA − 3T3, and CEA + LTK) were studied to determine whether or not the persistent feeder layer sensitizes the host to alloantigen. Mice grafted with CEA + LTK rejected second-set grafts at 12 days, with a tail graft survival curve nearly identical to mice receiving the flank autografts (Fig. 7). However, mice grafted with CEA + 3T3 and CEA − 3T3 demonstrated accelerated tail graft survival (9 and 10 days, respectively), compared to the negative control of 12 days ($p < 0.01$). Although the second-set survival curves suggest that CEA + 3T3 grafts may be more immunogenic than CEA − 3T3 grafts, the difference between these groups, and compared to the positive controls, is not statistically signifi-

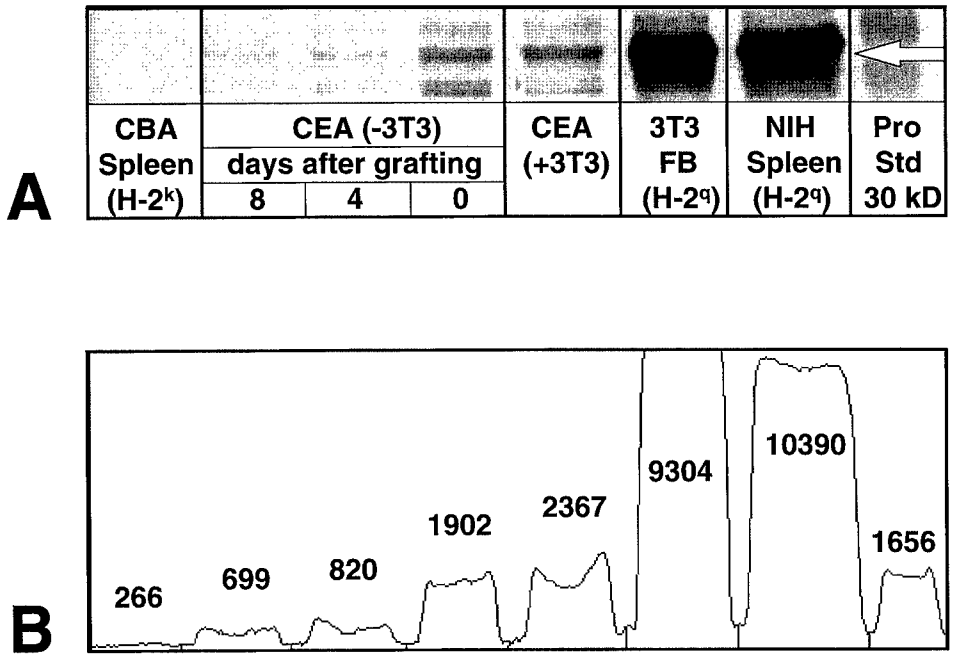

FIG 5. (*A*) Western immunoblot and (*B*) video densitometry depicting H-2^q class II antigen expression. Positive controls (NIH splenocytes and 3T3 fibroblasts, both H-2^q) express a 30-kd protein band (*arrow*) which represents the β-chain of H-2^q class II histocompatibility antigen. CEAs (H-2^k) co-cultured with 3T3s express significant H-2^q class II antigen both before and after differential trypsinization. H-2^q antigen, derived from 3T3s, persists in vivo after grafting but decreases after 8 days. Negative control CBA splenocytes (H-2^k) do not express any H-2^q class II antigen. Abbreviations: Pro Std, protein standard; FB, fibroblast; CEA, cultured epidermal autograft.

TABLE 2. Median survival times of second-set tail allografts

	Control Groups			Cultured Epidermal Autografts		
	NIH FT Allograft ($H\text{-}2^q$)	CBA FT Autograft ($H\text{-}2^k$)	3T3 IP ($H\text{-}2^q$)	CBA CEA +3T3 ($H\text{-}2^q$)	CBA CEA −3T3 ($H\text{-}2^q$)	CBA CEA +LTK ($H\text{-}2^k$)
MST, days	9*	12	9.5*	9*	10*	12
MST, range	7–11	10–14	7–12	7–11	7–12	10–14
N	16	15	16	10	15	13

MST, median survival time; N, number/group; FT, full-thickness; IP, intraperitoneal; CEA, cultured epidermal autograft.

* $p < 0.01$ vs. CBA FT, CEA + LTK groups.

cant. These data strongly imply that an allogeneic feeder layer, even when removed by conventional techniques, persists in vivo after CEA grafting, primes the host to alloantigen, and sensitizes for accelerated second-set rejection.

DISCUSSION

In this series of experiments, we provide evidence that allogeneic fibroblasts used to cultivate CEAs persist both in vitro and in vivo, despite initially inhibiting these fibroblasts with mitomycin-C and selectively removing them via differential trypsinization. Furthermore, we demonstrate that alloantigen from foreign fibroblasts can be detected in CEAs 8 days after application. Although foreign antigen from these fibroblasts apparently decreases over time, hosts are nonetheless sensitized to alloantigen and demonstrate accelerated rejection of second-set allografts.

Cultured keratinocyte grafts have been used for burn wound coverage for nearly 15 years, but no clinical trials have overwhelmingly proven the advantage of this biotechnology over more conventional methods of wound closure.[3–6,13] CEAs provide a potentially unlimited source of autologous epidermal cells and would appear to be an attractive solution to the problem of massive thermal injury and limited donor sites. However, graft preparation requires a cultivation period of 3 weeks,[14,15] delaying wound excision

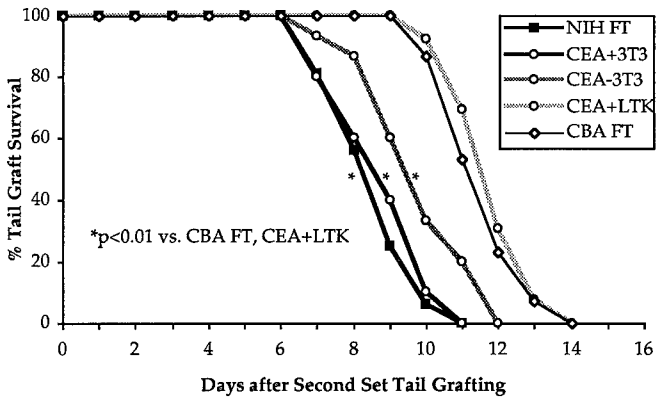

FIG 7. The effect of an allogeneic feeder layer of second-set rejection. CEAs cultivated with an allogeneic $H\text{-}2^q$ feeder layer (both removed and left intact) prime hosts for accelerated rejection of second-set tail allografts, compared to hosts receiving CEAs grown with a syngeneic $H\text{-}2^k$ LTK feeder layer ($p < 0.01$). Abbreviations: FT, full-thickness; CEA, cultured epidermal autograft.

and coverage and potentially mitigating the beneficial effects on host immunocompetence and survival.[16–20]

Another significant limitation of CEAs is graft fragility, which is owing not only to the lack of a dermal component, but also abnormal anchoring fibrils located in the basement membrane. These disorganized attachments occur in older, mature grafts, despite the formation of a neodermis.[21] Such fragility interferes with patient rehabilitation by delaying the onset of physical therapy and the application of compression garments. Other investigators are discouraged by CEAs because of their unpredictable initial graft take, requiring multiple operative applications to complete wound closure.[4,6] The significant expense of these grafts, estimated to be $13,000 per % total body surface area covered, also presents a problem for cost-effective wound resurfacing.[6]

In addition to the concerns listed above, several authors have reported the phenomenon of late graft loss, which occurs independently of graft infection or mechanical factors.[6–8] Clinically, patients develop an inflammatory response that has been described as "rejection," in which graft blistering results in multifocal destruction, ulceration, and occasionally complete graft loss. Rue and Pruitt noted that six of ten patients with >70% total body surface area burn sustained significant, late CEA loss that appeared to be immunologically mediated. We were initially concerned that xenogeneic proteins used as media supplements in keratinocyte cultures (fetal bovine serum (FBS), bovine pituitary extract) might persist as antigens on autogenous keratinocytes, sensitize the graft recipient, and initiate autograft destruction.[22,23] While we discovered that patients grafted with CEAs generate antibodies to FBS, we did not examine the correlation between late CEA breakdown and anti-FBS antibody titers.

However, another source of immunogenic protein may be the foreign fibroblasts used to enhance keratinocyte growth in vitro. Several investigators have proposed that persistent fibroblasts may interfere with functional studies of epidermal grafts[24] and even transmit oncogenes to the graft recipient.[15] Certainly, there is reasonable concern that xenogeneic fibroblasts, if not completely removed from CEAs before grafting, might initiate an antibody- and/or cell-mediated immune response that could jeopardize the fate of the entire graft.[4,15,25] Using both flow cytometry and Western immunoblotting, we recently reported that murine fibroblasts persist in human keratinocyte cultures after several passages and express significant amounts of class II histocompatibility antigens.[8] Based on the methodology of Rheinwald and Green,[9] CEAs had been generated by plating keratinocytes with a growth-arrested feeder layer that was later removed via differential trypsinization. Commercially available CEAs differ only in that these grafts have xenogeneic fibroblasts added to secondary and tertiary cultures to improve keratinocyte growth, according to the patent application. Fibroblasts may not be required to grow keratinocytes in vitro, but adding fibroblasts to epidermal cells in culture clearly accelerates confluence[15] and remains the standard practice of private industry in the generation of CEAs.

The focus of this study was to determine if these foreign

fibroblasts survived after CEA application to sensitize the host immunologically. To test whether or not feeder layer fibroblasts persist in vivo, we chose to grow murine keratinocytes from genetically pure donors (CBA mice, H-2^k haplotype) with allogeneic murine fibroblasts (3T3, H-2^q haplotype). CEAs were then grafted onto CBA hosts after reaching confluence. Western immunoblotting revealed the presence of class II alloantigen up to 8 days after grafting, despite employing conventionally accepted techniques of feeder layer removal. These results are consistent with our previous finding that such fibroblasts persist in vitro after several passages.[8] Fibroblasts in these grafts remain viable, can express considerable class II histocompatibility antigen, and may account for 3% of the final cell population.[8]

Quantifying long-term CEA survival in a murine model is difficult, because of the rapid wound contraction that occurs in mice. Therefore, to assess the immunogenicity of persistent alloantigen, we utilized the concept of second-set rejection, as originally described by Medawar.[12] We found that mice initially primed with FT allografts, IP 3T3 fibroblasts, or CEAs grown with 3T3s (independent of their removal) sensitized the hosts to alloantigen and resulted in the accelerated rejection of second-set tail allografts (compared to mice grafted with FT autografts or CEAs grown with syngeneic LTK fibroblasts). Selective removal of the allogeneic feeder layer resulted in slightly prolonged second-set allograft survival compared to the positive control, but this difference was not statistically significant and still resulted in a more vigorous second-set rejection response, compared to the negative control.

How trypsinized CEAs sensitize the host remains speculative, but most likely involves either the persistence of nonvisible 3T3s in vivo or the persistence of soluble antigen in the graft matrix. Although this represents a minimal amount of foreign antigen, a critical amount must remain in vivo, to be detected by Western immunoblotting 8 days after grafting. One would suspect that if enough antigen can be detected by such methods, then this same amount of foreign protein could sensitize the host immunologically and prime for accelerated second-set rejection. Our particular model, which involves an allogeneic system, is undeniably different from the human situation, in which keratinocytes are cultured with xenogeneic fibroblasts. However, we are confident that the alloantigen priming demonstrated in these experiments can be extended to xenogeneic sensitization. The ability to demonstrate such priming between two similar murine haplotypes implies that the greater histocompatibility mismatch between the human host and mouse fibroblast will yield at least equivalent sensitization to foreign antigen.

The delayed destruction of CEAs observed in some graft recipients may occur only after restoration of host immunocompetence. We have previously demonstrated that burn injury impairs alloantigen processing as a function of burn size.[26] Sensitization to alloantigen and the effector mechanisms designed to eliminate foreign antigen are both impaired following thermal injury. Therefore, patients may only generate an immunologic response to foreign fibroblasts in CEAs after wound closure, improvement in nutritional parameters, and restoration of immunocompetence. However, once priming has occurred, focal destruction directed at xenoantigens could possibly induce a generalized inflammatory response that results in total graft breakdown. Why some patients do not display this phenomenon can be explained by two possibilities: (1) the successful removal of CEA fibroblasts before grafting, or (2) the development of chimeric tolerance during host immunosuppression. Clearly, additional clinical trials are necessary to determine whether graft recipients develop antibodies to xenoantigen and/or generate a cytotoxic T lymphocyte response.

We conclude that the persistence of foreign fibroblasts in keratinocyte cultures limits the clinical utility of CEAs when used for wound coverage. Patients who require multiple applications, such as those with massive burn injury, may be particularly susceptible to antigen sensitization and, therefore, late CEA breakdown. While keratinocytes can be cultivated without the use of a fibroblast feeder layer, the addition of such cells clearly accelerates culture time and improves culture quality, perhaps through the production of eicosanoids, extracellular matrix, growth factors, or stimulatory cytokines.[4,15,27,28]

We are presently investigating alternative models of keratinocyte cultivation, which include the addition of potentially mitogenic, synthetic antibiotic peptides to the culture media, as well as the use of a plastic monomer that allows for the transfer of subconfluent keratinocytes, as early as 4 to 7 days after initial plating, to freshly excised wounds. However, if the presence of a fibroblast feeder layer proves to be essential for optimal keratinocyte expansion, then perhaps the insertion of a "suicide gene," designed to induce fibroblast apoptosis, may prove to be an effective method of feeder layer removal. Undoubtedly, such genetic manipulation, even if successful in eliminating foreign antigens, would only add to the cost of an already expensive technology. Improving the initial graft take, however, would decrease the need for successive applications and may actually decrease the overall cost of wound coverage.

The addition of autogenous fibroblasts to epidermal cultures might accelerate keratinocyte growth and improve graft quality, but current cell culture technology does not easily permit the creation of an immediately available fibroblast cell line.[29] Nevertheless, the incorporation of a dermal component remains critical to the success of cultured grafts. Composite skin substitutes utilizing autologous keratinocytes and fibroblasts seeded onto collagen substrates have been successful in recreating both epidermal and dermal elements but have not yet been studied extensively in clinical trials.[30]

Despite the inconsistent success of CEAs, cultured keratinocyte sheets are probably effective as a biologic dressing, capable of secreting growth factors and providing residual adnexal elements, such as sweat glands and hair follicles, the opportunity to repopulate the wound surface.[4] However, until CEAs can be grown without an immunogenic feeder layer and demonstrate clinical usefulness, we remain cautious about the widespread application of CEAs for burn wound coverage.

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DISCUSSION

Dr. David G. Greenhalgh (Cincinnati, Ohio): Dr. Hultman has presented another fine work from Dr. Meyer's laboratory that examines the immunologic consequences of using cultured epidermal autografts. The well-designed study examines the causes of late loss of cultured epidermal autografts in burn patients. The hypothesis is that the feeder layers of fibroblasts that are cocultured with the cultured epidermal autografts persist despite attempts to separate the fibroblasts from the cultured epidermal cells. The persistent fibroblasts sensitize the host and ultimately lead to loss of the cultured epidermal autograft.

The authors present convincing data that support the hypothesis, but I do have the following questions. The Western blot is a very sensitive test, but is it overly sensitive? It would be interesting to examine the wound by immunohistochemistry to see if indeed there are intact fibroblasts persisting in the autografts.

In a similar line using histology, is there an inflammatory reaction showing evidence of rejection in the mice at the time of rejection of their autografts?

The authors have shown pictures of the patients losing their cultured epidermal autografts. Have there been biopsies performed looking at histology for evidence of rejection in these patients?

I agree that many makers of skin substitutes use feeder layers from an allogeneic source. Since it takes 3 weeks for the cultured epidermal autograft to be prepared, why not use the patient's own fibroblast as a feeder layer? Even more simply, why not culture the keratinocytes in a fibroblast free

media, such as has been used on a regular basis by Dr. Boyce and other investigators?

Dr. Charles Cuono and later many other investigators, including Dr. William Hickerson, have described the use of fresh allografts for a temporary coverage of the burn wound. After 3 weeks, when cultured keratinocytes are ready, the epidermal portion of the allograft is debrided, leaving an allograft dermis on the wound surface. These investigators have clearly shown that covering the dermal allograft with cultured epidermal autograft is well tolerated, and there does not appear to be a rejection problem in these patients. Can you explain why there is no rejection to the use of a dermal allograft when you show that a possible cause for late loss of cultured epidermal autografts may result from persistence of fibroblasts of the feeder layer. I am certain that there would be persistent allogeneic fibroblast in the allogeneic dermis used by these investigators.

I agree that cultured epidermal autografts alone have been quite ineffective. They are prone to persistent blistering problems and scarring problems. Our natural skin is composed of a dermis and an epidermis, and most burn surgeons now feel that both components need to be used for adequate wound coverage. Even the originators of the cultured epidermal autograft techniques now believe that a dermal component is important. Should our studies with the use of cultured keratinocytes alone be abandoned?

Finally, many investigators have shown that there are still persistent and significant problems with the use of skin substitutes, whether using epidermal or dermal components alone, or with a composite skin made up of both a dermis and an epidermis. While these problems persist, investigations such as the one presented here are extremely important, because I feel that, at one point, the use of skin substitutes will become the mainstay of the treatment of burn patients. Thank you.

Dr. John F. Hansbrough (San Diego, California): These studies were very interesting. We need to remember, though, that if you use 3T3 feeder cells, which we do not, they are murine cells, and therefore applying them to the human is a heterologous transplant, not allogeneic as in your studies, which is really perhaps entirely different in that these cells would probably be rejected by the host so quickly they may not have any effect on their response.

So again, the human situation would be a heterologous response, not allogeneic as in your studies.

Dr. Kevin T. Farrell (Allentown, Pennsylvania): There is a relatively small window, between 7 and 10 days, between when there is no loss and everything is rejected, and the question that I would have, we are putting a great deal of emphasis on the difference between 1 to 2 days rejection, are the criteria for rejection such that we do not have or observe a bias in commenting on this?

Dr. Basil A. Pruitt, Jr. (San Antonio, Texas): Is it possible to eliminate the allogeneic fibroblasts by exposure to a more concentrated solution of trypsin or exposure to the trypsin for a longer duration of time?

Secondly, in your clinical practice, have you noticed that with repeated applications of cultured cells there is greater and greater loss because of this phenomenon?

Thirdly, we know that with chimeric syngeneic, allogeneic constructs of cultured cells, there is retention and just gradual replacement of the allogeneic cells. How do you reconcile your findings with that phenomenon?

Dr. C. Scott Hultman (closing): Thank you very much for all of those comments. I will try to address each of the questions one by one.

First of all, Dr. Greenhalgh underscored the most important goal of our study, to create a permanent biologic skin replacement, which has properties that approach those of regular skin. Until we can perfect the dermal components of these cultured skin substitutes, we will be less than completely satisfied with these skin replacements.

Dr. Greenhalgh mentioned that the Western blot may not be the best assay to detect the presence of foreign protein, given its problems with specificity and sensitivity, but in our experiments, we found very little cross-reactivity with the $H\text{-}2^k$ antigen compared to the $H\text{-}2^q$ alloantigen. We are confident that the KL295 monoclonal antibody is not overly sensitive.

Secondly, Dr. Greenhalgh asked about immunohistochemistry, and we do not have any biopsy samples examining the persistence of these fibroblasts in vivo. But certainly that would be something we would want to pursue in future investigations.

Thirdly, Dr. Greenhalgh asked if we had obtained any biopsies of those patients who demonstrated autograft "rejection." The slide of the patient with autograft breakdown is actually 5 years old and does not have any accompanying histology. At that time, we suspected that this phenomenon was immunologically mediated, but we had no understanding of the mechanism. We abandoned the use of cultured epidermal autografts clinically and decided to focus our laboratory efforts on the basic science of trying to create a long-term skin replacement: accelerating keratinocyte growth in vitro, removing foreign antigens from the culture process, and studying the effect of burn immunosuppression on antigen processing.

Dr. Hansbrough expressed some concern that our model involved allogeneic fibroblasts, when in fact xenogeneic cells are used in commercially available skin grafts. In order to simplify our model, we chose to work with an entirely murine system. We felt that if we could show priming with allogeneic fibroblasts, then certainly the presence of xenogeneic fibroblasts would induce at least as strong a rejection response, if not greater, although we do not have the data to back that up.

Dr. Farrell asked about observer bias between the groups in terms of identifying second-set rejection of these tail grafts. We utilized a model that was reported several decades ago and represents a fairly standard approach to assess priming and sensitization, at least in the immunologic literature. Furthermore, we had two separate observers scoring the rejection response, and we have replicated our findings over the course

of several different studies. Our group is very confident that this particular model of second-set rejection is reliable and accurate.

And finally, to address Dr. Pruitt's questions, I think he raises an excellent, provocative concept of chimerism, which basically represents the incorporation of nonself into self through various immunologic mechanisms, either through tolerance or through host nonreactivity. Burn patients that are immunocompromised may have a higher propensity for chimerism than the immunocompetent host.

In this particular experiment, our hosts were immunocompetent, and were able to mount an immunologic response to these foreign fibroblasts. In burn patients, however, the prolonged survival of cultured epidermal allografts or autografts grown with foreign fibroblasts may be due to this chimerism that Dr. Pruitt has suggested. This might explain why many patients do very well with these cultured skin substitutes and do not have this rejection phenomenon that we have observed in a small subgroup of patients.

Finally, Dr. Pruitt asked whether or not prolonged trypsinization of cultured epidermal sheets might be more effective in removing the fibroblast feeder layer. In our experience, extended exposure to this enzyme damages the grafts and limits their effectiveness as a biologic skin replacement.

I think that wraps it up. Thank you very much.